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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US97/21272 <b>(22) International Filing Date:</b> 26 November 1997 (26.11.97)  <b>(30) Priority Data:</b> 08/756,693      26 November 1996 (26.11.96)      US  <b>(71) Applicant (for all designated States except US):</b> BAYLOR COLLEGE OF MEDICINE [US/US]; Room 186A, One Baylor Plaza, Houston, TX 77030 (US).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> LUNDBLAD, Victoria [-/US]; 1912 Vermont Street, Houston, TX 77019 (US).  <b>(74) Agents:</b> KOCH, Robert, J. et al.; Fulbright & Jaworski L.L.P., 801 Pennsylvania Avenue, N.W., Washington, DC 20004 (US).	<b>(81) Designated States:</b> AU, CA, JP, KR, MX, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>Without international search report and to be republished          upon receipt of that report.</i>	
<b>(54) Title:</b> IDENTIFICATION OF INHIBITORS THAT PREVENT ACCESS OF TELOMERASE TO CHROMOSOMAL TERMINUS  <b>(57) Abstract</b>  <p>The present invention discloses novel telomerase-associated proteins as well as methods for isolating the genes encoding telomerase-associated proteins. Using novel screening methods presented additional telomerase-associated genes may be isolated and their protein products expressed and characterized. Identification of protein-protein interactions required for the <i>in vivo</i> function of telomerase provides the basis for a novel screening method for anti-tumor agents.</p>		

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## IDENTIFICATION OF INHIBITORS THAT PREVENT ACCESS OF TELOMERASE TO CHROMOSOMAL TERMINUS

### Field of the invention

5           The present invention relates to the fields of cellular and molecular biology. In particular, the present invention relates to genes encoding proteins that play a role in telomerase function and regulation.

### Background of the invention

10           Telomeres are specialized sequences present at the ends of linear eukaryotic chromosomes, and are required for genomic stability and complete replication of chromosomal termini (reviewed in Blackburn and Greider 1995; Zakian 1995; Greider 1996). Telomeres are characterized by a tandem array of short repeated DNA sequences; in humans, the telomeric repeat sequence  
15 is a d(TTAGGG)<sub>n</sub>.

          In all eukaryotes examined to date, with the exception of *Drosophila* and several other dipterans, telomeres are replicated by a specialized DNA polymerase called telomerase (reviewed in Kipling 1995; Blackburn and Greider 1995; Zakian 1995). This enzyme is responsible for adding telomeric  
20 repeats onto the 3' end of the G-rich strand of the telomere, and uses an internal template present in an RNA subunit to dictate the sequence of the added telomeric DNA. The gene encoding the RNA component of telomerase has been identified in a number of species (Greider and Blackburn 1989; Shippen-Lentz and Blackburn 1990; Lingner, *et al.* 1994; MEachern and  
25 Blackburn 1995; Feng, *et al.* 1995; Blasco, *et al.* 1995), including the *TLC1* gene from *S. cerevisiae* (Singer and Gottschling 1994). However, in contrast, the characterization of the protein components of this enzyme has lagged behind. In addition, little is known at a molecular level about how regulation of telomerase activity is achieved.

30           There are several approaches to identifying additional components of the telomerase enzyme complex. Biochemical purification of telomerase from the ciliate *Tetrahymena* has recently led to the identification of two proteins which, in addition to the RNA component, comprise subunits of the enzyme (Collins, *et al.* 1995); homologs of these genes have not been identified yet in

other species, including in the completely sequenced *S. cerevisiae* genome. An alternative to the biochemical route is to identify genes which, when mutated, have a set of phenotypes predicted for a defect in telomerase *in vivo*. These two different protocols presumably could lead to identification of at least some of the same factors. However, a genetic approach has the potential to identify components that may be critical for *in vivo* function (for example, as positive regulators) but which are not required *in vitro* for enzymatic activity and therefore may not co-purify with the enzyme.

Two genes have been previously identified in *Saccharomyces cerevisiae* which, when deleted, result in a cell with phenotypes expected from the elimination of telomerase activity. The first of these is the *EST1* gene, which encodes a highly basic 82 kD protein hypothesized to be a subunit of telomerase (Lundblad and Szostak 1989; Lundblad and Blackburn 1991). This proposal has been based largely on the observation that *est1* null mutant strains display two characteristics predicted for a telomerase deficiency: progressive loss of telomere sequences from chromosomal termini and a senescence phenotype (manifested as a steady decline in cell viability). Strong support that such phenotypes could be diagnostic for a defect in telomerase came from the demonstration that a yeast strain deleted for the *TLC1* gene, encoding the yeast telomerase RNA, exhibits the same set of phenotypes (Singer and Gottschling 1994).

Although telomerase activity is still present in chromatographic fractions prepared from an *est1-Δ* strain, suggesting that Est1p is not required *in vitro* for catalytic activity (Cohn and Blackburn 1995), this does not exclude the possibility that *EST1* is a non-catalytic component of telomerase (Lin and Zakian 1995). Since the Est1 protein binds single-stranded yeast telomere sequences Est1 may function as a telomerase component responsible for telomere recognition. Consistent with this, Est1 protein is associated with the telomerase RNA and/or enzyme activity (Lin and Zakian 1995; Steiner, *et al.* 1996), although it was not possible to determine whether the association was quantitative in these experiments. The properties of Est1 binding to telomeric DNA are also consistent with a role as a telomere-end-binding protein acting as a positive regulator of telomerase function by directing telomerase onto the chromosomal terminus. In either model, the requirement

for Est1 function might be obviated in the *in vitro* experiments in which naked DNA is used as a substrate for telomerase elongation.

In mammalian cells, telomerase is highly regulated: it is present in the germ line but is absent or greatly reduced in most normal tissues. Consistent with the absence of this enzyme, telomeres shorten in these normal cells. During the process of tumor formation, telomerase activity is up-regulated, relative to the levels present in normal cells. This up-regulation is presumed to be necessary in order to maintain telomere length and allow unregulated proliferation during tumor development.

This proposal is supported by a substantial survey of the status of enzyme activity in different tissues: telomerase activity is expressed in 851 out of 998 human tumor tissues, but is not present in most normal tissues, with the exception of the germ line and hematopoietic stem cells (Shay and Wright 1996, and references therein). In some tumors, telomerase is expressed at higher levels in late-stage cancers as compared to early-stage cancers, with some data suggesting that the survival rate of patients correlated with the levels of telomerase activity (Hiyama, *et al.* 1995a, 1995b).

Specific studies of human breast cancer have also demonstrated a strong correlation between tumor development and telomerase activity, as had been predicted on the basis of an analysis of the rate-limiting step in most metastatic breast cancers (Shay, *et al.* 1993). Initial data demonstrated that alterations in telomere length occurred in established breast cancer cell lines, and the extent of the change in telomeric tract length correlated with the histologic aggressiveness of breast carcinoma (Rogalla, *et al.* 1994; Odagiri, *et al.* 1994). Subsequently, a direct analysis of telomerase activity showed a striking correlation between enzyme activity and cancer progression; activity was detected in > 95% of advanced stage breast cancers, whereas it was present in 68% to 81% of less advanced tumors and absent in fibrocystic disease (Hiyama, *et al.* 1996).

Collectively, this data strongly argues that the reactivation of telomerase is a necessary event for sustained tumor growth. Therefore, anticancer agents that target this enzyme may provide a new approach to cancer treatment, including breast cancer (Harley, *et al.* 1995). In addition, because telomerase expression is almost exclusively restricted to tumor cells,

this suggests that telomerase inhibition may have limited side effects when used as an anti-cancer therapeutic. The limited population of stem cells which are telomerase-plus and which would therefore be affected by telomerase inhibitors compares favorably to the much wider range of proliferating normal tissues that are the unintended target of most  
5 chemotherapy agents. Thus, inhibition of telomerase has been proposed as a potential anti-cancer therapeutic target, with the presumed advantage that inhibition of telomerase may have little side-effects, since it may not be needed for normal somatic cell growth.

10 Currently, most efforts are directed at trying to find compounds that inhibit the catalytic activity of the enzyme, defined as the *in vitro* polymerase activity. However, several observations suggest that telomerase activity, defined as the maintenance of a functional telomere, can also be regulated at levels other than the catalytic activity of the enzyme. Telomerase catalytic  
15 activity is present in certain human cell lines such as hematopoietic cell lines (Broccoli, *et al.*, 1995; Counter, *et al.*, 1995) which nevertheless display telomere shortening (Cooke and Smith 1986; Allshire, *et al.*, 1988; de Lange, *et al.*, 1990). This argues that other factors must also be involved in mediating telomere length control, presumably by regulating telomerase  
20 activity in some way. However, identification of these components in mammalian systems has been problematic, due to the absence of an assay system for their detection.

## SUMMARY OF THE INVENTION

25 We have approached the problem of identifying telomerase components by searching for such factors in yeast. Specifically, we were interested in looking for mutants of yeast that exhibited the phenotypes predicted for a defect in telomerase (telomere shortening and a senescence phenotype).  
30 Strong support that these characteristics would be diagnostic for a telomerase defect came from the demonstration that a strain deleted for the yeast telomerase RNA gene, *TLC1*, also showed these same two phenotypes (Singer and Gottschling 1994).

Our screen identified four genes; the previously identified *EST1* gene (Lundblad and Szostak 1989) and three new genes (Lendvay, *et al.*, 1996). Genetic analysis demonstrated that mutations in these four genes disrupted the same pathway for telomere replication as a mutation in *TLC1*, a known component of telomerase (Lendvay, *et al.*, 1996).

Three of these genes (*EST1*, *EST2* and *EST3*) are novel genes that function solely in the same pathway for telomere replication as defined by *TLC1* (Lendvay, *et al.*, 1996). However, we have shown that the fourth gene (initially called *EST4* but subsequently shown to be the same as the previously cloned *CDC13* gene) has a dual role in telomere function (Nugent, *et al.*, 1995). The *CDC13* gene is an essential yeast gene that is required for maintaining telomere integrity, as first suggested by a study from Lee Hartwell's laboratory showing that a rapid loss of one strand of the telomere occurs in the absence of *CDC13* function (Garvik, *et al.* 1995). Recent work from our laboratory has uncovered an additional role for Cdc13 in telomere maintenance. This resulted from the discovery of a novel mutation, called *cdc13-2<sup>est</sup>*, that displayed a phenotype virtually identical to that of a telomerase-minus strain (although, as just discussed, enzyme levels were normal in extracts prepared from this strain). Furthermore, epistasis analysis between the *cdc13-2<sup>est</sup>* mutation and the *tlc1-Δ* mutation demonstrated that *cdc13-2<sup>est</sup>* perturbs a function of *CDC13* that is required for the telomerase pathway.

In contrast, genetic analysis of the previously isolated conditional lethal allele of *CDC13* (*cdc13-1<sup>le</sup>*) showed that *CDC13* has a separate essential function that must be maintained in addition to the telomerase-based pathway. We have also shown that purified *CDC13* protein binds specifically to single-stranded yeast telomeric substrates. Based on these data, we proposed that *CDC13* has two distinct functions at the telomere (Nugent, *et al.* 1996). The first of these proposed roles is to protect the end of the chromosome, which is essential for cell viability. Second, we proposed that Cdc13 protein regulates telomerase by mediating, either directly or indirectly, access of this enzyme to the chromosomal terminus, and that this access is now eliminated by the *cdc13-2<sup>est</sup>* mutation.



The present invention provides the primary structure of two new telomerase-associated proteins Est2 and Est3.

An object of the present invention is to provide a method for identifying and isolating other telomerase-associated proteins using the Est2 or Est3 proteins or fragments thereof.

An object of the present invention is to provide a method for identifying and isolating other telomerase-associated proteins using the Est2 or Est3 proteins or fragments thereof to generate antibodies and to use the antibodies thus generated to purify telomerase-associated proteins.

An object of the present invention is to provide a method to identify telomerase-associated proteins using standard biochemical techniques.

An object of the present invention is to provide a novel method for screening for *EST* genes.

An object of the present invention is to provide an assay for the identification of a novel class of telomerase inhibitors that function through a mechanism not taught by the prior art.

An object of the present invention is provide a method for finding the human homologues of yeast telomerase-associated proteins by identifying conserved motifs in the yeast telomerase-associated proteins and searching human protein databases for such conserved motifs.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of the experimental protocol used to isolate the genes coding *est* mutations.

Figure 2A shows a Southern blot analysis of telomeric DNA of yeast strains having mutations in the *EST* genes.

Figure 2B shows the results of successive streak-outs of mutant yeast strains.

Figure 3A shows a Southern blot analysis of telomeric DNA of yeast strains having mutations in the *EST* genes.

Figure 3B shows the results of a viability assay of yeast strains with various mutations.

Figure 4A shows a Southern blot analysis of yeast strains with various mutations.

Figure 4B shows a Southern blot analysis of yeast strains with various mutations.

5 Figure 4C shows the results of a viability assay of yeast strains with various mutations.

Figure 5A shows a schematic representation of the strategy used to locate the *EST2* open reading frame.

Figure 5B shows the primary structure of the *EST2* gene product.

10 Figure 5C shows the results of a viability assay of yeast strains with various mutations.

Figure 6 shows the primary sequence of the *EST3* gene product.

Figure 7 shows the nucleotide sequence of the *EST3* gene including flanking regions and location of the +1 ribosomal frame shift.

15 Figure 8 shows the transposon mapping of the *EST3* gene.

Figure 9 shows a model for ribosome frame shifting in *EST3*.

Figure 10A-10H show various *EST3* constructs.

Figure 11 shows the structure of the *EST3* gene.

20 Figure 12 shows a plasmid map of a fusion construct of the *EST2* protein.

Figure 13 shows a plasmid map of a fusion construct of the *EST2* protein.

Figure 14 shows the effect on viability of a mutant strain containing *cdc13-1<sup>Δ</sup>* and *tlc1Δ*.

25 Figure 15A shows a schematic representation of the structure of the Est3-fusion proteins constructs prepared and Figure 15B shows a Western blot analysis of the expression of the protein constructs.

## DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

30

## MATERIALS AND METHODS

**Yeast strains and media:** *S. cerevisiae* strains used in this study are shown in Table 1; YPH275 was the diploid parent of all strains used in these studies. The mutant derivatives MVL1 to MVL26 were isolated, as

described below, from the haploid strain TVL227-1A, obtained by transformation of YPH275 with plasmid PVL106 (Lundblad and Szostak 1989) and subsequent sporulation and dissection. TLV120 and TLV140 were described previously (Lundblad and Szostak 1989; Lundblad and Blackburn 1993); DLV131 was constructed from YPH275 by introducing a *tlc1-Δ::LEU2* disruption, constructed by PCR-mediated deletion (Baudin, *et al.* 1993) of sequences between 192 and 909. Strains were grown at 30 °C and in standard media (Guthrie and Fink 1991), except that chromosome loss assays were performed on media with limiting adenine (6 μg/ml) to enhance the detection of red Ade<sup>-</sup> sectors in colonies. Canavanine plates and 5-FOA SC-LEU plates were prepared as described in Ausubel, *et al.* 1987. Yeast transformations were performed using the lithium acetate method (Schiestl and Gietz 1989); sporulation and tetrad dissections were carried out using standard techniques (Guthrie and Fink 1991).

**EMS Mutagenesis:** TVL227-1A was grown overnight to saturation in SC-LEU-TRP, diluted ten-fold into YEPD media and grown to O.D. 0.8 to 1.0. Forty ml of cells were washed twice in sterile dH<sub>2</sub>O and re-suspended in 12 ml 0.1M sodium phosphate, pH 7.0; 1.7 ml portions were incubated with 45 μl of EMS (ethylmethane sulfonate) for one hour at 30 °C with slow aeration. Cells were diluted 10-fold in sterile 5% sodium thiosulfate, washed twice in 5% sodium thiosulfate, twice in sterile dH<sub>2</sub>O and re-suspended in 2.0 ml dH<sub>2</sub>O. Aliquots were plated on canavanine plates to monitor the increase in frequency of canavanine resistance and dilutions plated on YEPD plates to determine the percent of cells that survived the mutagenesis procedure; both values were determined relative to a sample of cells handled in parallel but without added EMS. Because of the large number of colonies screened, the entire screen was performed in three stages, with each stage handling between 80,000 and 150,000 colonies. A total of 12 independent mutageneses were performed; the average survival ranged from 30 to 60% and the increase in canavanine resistance was 50 to 150-fold. Note that since all screening steps were performed at 30 °C, this mutant screen was not specifically designed to recover conditional lethal mutations.

**Mutant screen:** *Colony sectoring screen:* To detect mutants which show a phenotypic delay in chromosome loss, mutagenized cells were processed through two rounds of growth as single colonies. After mutagenesis, cultures were plated on SC-LEU-TRP plates at a density of 500 - 1000 per plate and colonies allowed to grow at 30 °C to full size, for approximately three to four days. Pools of ~ 7500 colonies were generated by re-suspending colonies from SC-LEU-TRP plates in sterile dH<sub>2</sub>O, and re-plating at a density of ~ 350 per plate on SC-LEU plates with limiting adenine. The total number of colonies re-plated from each pool was approximately four-fold the size of the pool, to increase the odds that each colony in the original pool was represented. Plates were incubated for seven days at 30 °C to allow full development of red Ade<sup>-</sup> sectors; in addition, overnight incubation at 4 °C prior to scoring for chromosome loss helped enhance detection of red sectors (F. Spencer, personal communication). If two *est* mutants were isolated from a single pool and were later shown to map to the same complementation group, they were considered to be siblings of each other and only one was subsequently analyzed.

*Plasmid linearization assay:* Colonies with increased numbers of Ade<sup>-</sup> sectors were picked into microtiter dish wells containing 0.2 ml SC-LEU media, to select for retention of pVL106 (Lundblad and Szostak 1989). After two days growth at 30 °C, 5 µl aliquots from each well were transferred to SC-LEU-5-FOA plates, using a multiple-channel pipettor, and plates were subsequently incubated 3 more days at 30 °C to allow the growth of 5-FOA resistant micro-colonies. A wild type un-mutagenized strain gives rise to ~ 10-15 micro-colonies on the 5-FOA plates in this assay; all mutants that showed more than a three-fold reduction were saved for further analysis. Each candidate was recovered from the microtiter dish well and streaked for single colonies on SC-LEU plates, and three single colonies for each were re-tested in the plasmid linearization assay. Candidates which passed this re-test were assayed for alterations in telomere length by Southern analysis. In addition, candidates that reached this stage were transferred to 15% glycerol and stored at 70 °C, to prevent the loss of rapidly senescing strains.

*Telomere length analysis:* One to two single colonies from SC-LEU streak-outs for each candidate that passed the plasmid linearization assay were grown to saturation in 8 ml YEPD and genomic DNA prepared as described previously (Guthrie and Fink 1991). Samples of ~1 µg of DNA digested with *Xho*I enzyme (New England Biolabs) were separated on 20 cm 0.7% agarose gels, transferred to nylon membranes and probed with poly d(GT/CA), as described previously (Lundblad and Szostak 1989). When telomere length was assayed with increasing numbers of generations of growth (Figures 2 and 4), cultures were grown to saturation in YEPD and subsequently diluted by a factor of 10<sup>-4</sup> and allowed to re-grow to saturation; this process was repeated three to four times.

*Cellular senescence assays:* All mutants with decreases in telomere length of >250 bp were tested for whether they exhibited an associated senescence phenotype by back-crossing each mutant to a wild type haploid derivative of YPH275 and sporulating and dissecting the subsequent diploid strain. For each mutant, haploid spore products from four to six tetrads were streaked for single colonies on YEPD, incubated at 30 °C for 48 hours and scored for growth characteristics in parallel with *est1-Δ1::HIS3* haploid spore products generated from a dissection of TVL120. Single colonies from these "1X" streak-outs were subsequently re-streaked and similarly analyzed; this process was repeated up to four times. For the data presented in Figures 2, 3 and 4, each of these successive YEPD streak-outs were stored at 23 °C and used to re-assemble a time course on a single plate.

*Genetic manipulations: Complementation analysis:* Diploids were isolated from un-selected matings, to prevent selection for reversion or suppression of the senescence phenotype. Haploid strains of opposite mating type were patched together on YEPD plates and allowed to mate for four to six hours. Four zygotes were picked for each cross by micromanipulation with a Zeiss dissecting microscope and allowed to grow at 30 °C for two days. The resulting strains were purified once via single colony isolation and tested for mating type and any nutritional markers that differed between the two haploid parents. To assay for complementation of telomere length, one single colony from two to three

diploids were grown up and assayed by Southern analysis along with each of the two haploid parents. The growth characteristics and chromosome stability of multiple diploids for a given cross were similarly analyzed in parallel with the haploid parents.

5            *Linkage analysis and construction of double mutant haploid strains:*

Haploid *est* spores of opposite mating type were used to generate diploids (also by un-selected matings) which were heterozygous for two different *EST* genes. Diploids were sporulated and dissected and spores analyzed for cellular senescence and telomere length. Initially, both tetrads with  
10 four viable spores as well as tetrads with less than four viable spores were analyzed; when no evidence for synthetic lethality was obtained (see Figure 4), subsequent analysis concentrated on tetrads with four viable spores. The following crosses were performed to demonstrate that the new complementation groups (*EST2*, *EST3* and *EST4*) were distinct from each  
15 other and from *EST1* and *TLC1*: (i) *est2-1* x *est1-Δ::HIS3*; (ii) *est2-1* x *tlc1-Δ::LEU2*; (iii) *est2-1* x *est3-1*; (iv) *est3-1* x *est1-Δ::HIS3*; (v) *est3-1* x *tlc1-Δ::LEU2*; (vi) *est4-1* x *est1-Δ::HIS3*; (vii) *est4-1* x *tlc1-Δ::LEU2*. For each cross, ≥ 8 tetrads were analyzed for telomere length and senescence, with at least four tetratype and non-parental ditype tetrads recovered and  
20 analyzed for each. The resulting diploid strains were also used to construct the double and single mutant haploid strains analyzed in Figure 4. The *est2-1*, *est3-1* and *est4-1* haploids used to generate these diploids were the result of at least three backcrosses to wild type, and the *est1-Δ::HIS3* and *tlc1-Δ::LEU2* haploids were isolated from TLV120 and  
25 DVL131, respectively.

*Identification of est survivor strains:* Each freshly generated *est* haploid strain was grown in liquid YEPD culture, with successive dilutions, for 120 to 150 generations and subsequently plated for single colonies on YEPD plates. After two days growth at 30 °C, large colonies were  
30 examined for both growth phenotype and telomere structure on Southern blots, as described above.

**Cloning of the *EST2* gene:** Freshly dissected *est2-1* mutant strains were transformed with genomic libraries and screened for complementation of the senescence phenotype, using two approaches:

(1) An *est2-1 rad52::LEU2* strain was transformed with three different *URA3 CEN* genomic libraries (Rose, *et al.* 1987), with approximately 2000 transformants recovered for each transformation (which probably was a substantial under-estimate of the transformation efficiency, due to the severe growth defect of the *est2-1 rad52::LEU2* strain). Complementing clones were not apparent among any of these primary transformants; however, upon scraping and re-plating, healthy colonies were identified from two of the three library transformations. One single colony from each library pool was saved for future analysis; both were shown to have wild type telomeres as assayed on Southern blots.

(2) An *est2-1* strain at an intermediate stage in the senescence progression was transformed with a 2  $\mu$  *LEU2* library (Engebrecht, *et al.* 1990) and ~30,000 transformants were carefully screened after 40 to 45 hours for those transformants with a slight growth advantage. Fifty-seven candidates were selected and streaked for single colonies twice in succession to test for complementation of the senescence phenotype. Ten of the 57 candidates showed a healthy growth phenotype by this test; four also had wild type telomere length and were saved for further analysis (the remainder demonstrated the recombination-dependent telomere bypass pathway previously observed for *est1* mutants; Lundblad and Blackburn 1993).

For the six transformants recovered from the above three library transformations, the healthy growth phenotype and wild type telomere length were shown in each case to be plasmid-dependent. Plasmids were subsequently recovered from each yeast strain by rescue through *E. coli*. A combination of restriction mapping and Southern analysis of the genomic inserts demonstrated that the four plasmids recovered from the 2  $\mu$  *LEU2* library were identical to each other, and had genomic inserts that overlapped with the inserts present in the two plasmids recovered from the *URA3 CEN* genomic libraries. A 4.4 kb sub-cloned fragment common to these three unique plasmids capable of complementing *est2-1* was subjected to insertional mutagenesis using the bacterial transposon 8.7  $\delta$  (Strathmann, *et al.*, 1991). Insertion mutations were mapped by PCR, using primers against the transposon and polylinker sequences that

bounded the cloned insert. The  $\gamma\delta$  insertions subsequently provided the basis for sequence analysis, using primers unique to the right and left ends of the 8.7 alt 235 transposon; sequence was confirmed by comparison to that generated by the Yeast Genome Sequencing Project. The *est2-*  
5  *$\Delta 1::URA3$*  disruption mutation was constructed by deleting an internal 2.2 kb *HindIII* fragment, removing aa 13 to aa 686 of the *EST2* ORF, and inserting a 1.17 kb *URA3 HindIII* fragment. This disruption was introduced into TVL140 to generate an *EST2/est2- $\Delta 1::URA3$*  heterozygous diploid (strain TVL228), which was confirmed by Southern analysis.  
10 Twelve tetrads were analyzed in detail for senescence and telomere phenotypes, as described above.

### EXAMPLE 1

15 Identification of an expanded collection of *S. cerevisiae* mutants with a telomere replication defect and a senescence phenotype.

The original identification of the *est1-1* mutant employed a screen that assayed the behavior of a circular plasmid containing inverted repeats of *Tetrahymena* telomeric sequences bracketing the yeast *URA3* gene (Lundblad and Szostak 1989). At a low frequency in wild type cells, this circular plasmid can be converted into a stable linear form. This conversion requires  
20 the addition of yeast telomeric sequences onto the *Tetrahymena* termini in order to form functional telomeres. Since the conversion to a linear results in the loss of the *URA3* gene, mutants defective in the linearization process can be identified by screening for colonies that exhibit decreased frequencies  
25 of resistance to the drug 5-FOA (*Ura*<sup>+</sup> cells convert 5-FOA to a toxic intermediate, whereas the loss of *URA3* prevents this; Boeke, *et al.* 1984). Potentially, one subset of mutants that could be identified by this assay would also be defective for the ability to form functional telomeres, once the plasmid was linearized; such mutants should also exhibit shorter chromosomal  
30 telomeres. From this original screen, the *est1-1* mutant was the one candidate that fell into this class, with a progressive reduction in telomere length with increasing growth of the culture (Lundblad and Szostak 1989). However, one drawback to the plasmid linearization assay was that the process of testing individual colonies for their frequency of 5-FOA resistance



was labor-intensive, which limited the original screening to only ~7000 mutagenized colonies.

Once identified, both the *est1-1* mutant and the subsequently constructed *est1-Δ* strain were shown to have two additional associated phenotypes: a senescence phenotype, manifested as a gradual decrease in cell viability, and an accompanying progressive increase in frequency of chromosome loss (Lundblad and Szostak 1989). In order to isolate additional *est*-like mutants from yeast, all four of the phenotypes displayed by *est1* mutants were incorporated into an expanded screening protocol, presented in Figure 1. Due to the difficulty in screening large numbers of colonies in the plasmid linearization assay, this step was first preceded by an enrichment step for colonies that exhibited increased frequencies of chromosome loss. Candidates which showed both an increase in chromosome loss and a defect in the plasmid linearization assay were subsequently screened for alterations in telomere length by Southern blot analysis. Finally, those isolates with short telomeres were tested for an associated senescence phenotype, by examining freshly generated haploid mutants for whether they showed the characteristic decline in viability over time previously observed in *est1* and *tlc1* mutants.

Due to one unique aspect of the effect of *est1* mutations on chromosome instability, the standard chromosome loss assay used by others required modification. Detection of alterations in chromosome stability relied on a previously developed color-based visual assay that monitored the presence/absence of a non-essential 150 kb artificial test chromosome (Spencer, *et al.* 1990). A number of screens have been performed which use this assay, or similar variants, to detect mutants which affect chromosome fidelity (Meeks-Wagner, *et al.* 1986; Spencer, *et al.* 1990; Kouprina, *et al.* 1988; Runge and Zakian 1993). None of these previous screens, which have collectively identified a large number of genes involved in chromosome maintenance, detected mutations in either *EST1* or the new *EST* genes identified in this work. This is presumably due to the fact that the effect of *est1* mutations on chromosome loss shows phenotypic lag; no substantial increase in chromosome instability in an *est1* mutant strain is observed until approximately 40 - 60 generations of growth (Lundblad and Szostak 1989).

Therefore, to screen for mutants which showed similar delayed effects on chromosome stability required outgrowth after mutagenesis. Since the desired class of mutants was also expected to have a senescence phenotype, growth in liquid would exert a substantial selective disadvantage for such mutants. To prevent this, mutagenized cultures were processed through two rounds of growth as single colonies prior to screening for chromosome instability (see Materials and Methods for more details).

A total of 350,000 single colonies from 12 independently mutagenized cultures of yeast were screened by the four-tiered system shown in Figure 1. Individual colonies from cultures mutagenized with EMS to an average survival of ~50 % were processed in batches of 80,000 to 150,000 colonies. The first two steps (chromosome loss and the plasmid linearization assay) resulted in enrichment steps of approximately 20-fold and 50-fold, respectively. Southern blot analysis of telomere length of 375 candidates that passed these two tests led to the identification of 49 mutants with either decreases (ranging from ~50 bp to >300 bp) or increases (ranging from 150 bp to > 2 kb) in telomere length. Of the 36 mutants with shorter telomeres, 19 also had an associated senescence phenotype. These 19 *est* mutants, plus three additional short telomere mutants which had no detectable senescence phenotype but were subsequently shown to map to *EST* genes, are the subject of this report.

**The new *est*<sup>-</sup> mutants map to four genes:** Each of the *est* mutants, when crossed to wild type, were recessive for telomere length, chromosome loss and senescence (data not shown). The 19 mutants were also tested for whether they contained mutations in either *EST1* or *TLC1* by crossing each mutant to an *est1*-Δ strain and an *tlc1*-Δ strain; the resulting diploids were examined for both telomere length and viability. All 19 mutant strains fully complemented both phenotypes of the *tlc1*-Δ mutation, indicating that no new alleles of *TLC1* had been identified (data not shown). However, eleven mutants failed to complement both the senescence and short telomere phenotypes of the *est1*-Δ strain. Two additional mutants, with short telomeres but no obvious senescence phenotype, were also subsequently shown by complementation analysis to contain weak *est1* mutations. This complementation data was confirmed for five of these new *est1* alleles by

demonstrating that the mutation could be gap-repaired onto an *EST1*-containing plasmid with a gap encompassing the wild type *EST1* gene (C. Nugent and V. L., unpublished data). The identification of 13 *est1* alleles demonstrated that this expanded screen was capable of detecting *est* mutant strains.

The remaining eight *est* strains were crossed against each other in pairwise combinations and the resulting diploids were analyzed for telomere length, chromosome loss and senescence. This analysis indicated that these eight mutants defined three new complementation groups: *EST2*, *EST3* and *EST4*. Four mutations mapped to *EST2*, three mapped to *EST3* and one isolate mapped to *EST4*. One additional mutant, with short telomeres but an apparently wild type growth phenotype, was subsequently shown to contain a non-senescent allele of *EST3*. To confirm the complementation analysis, linkage analysis was performed on one to two isolates from each new complementation group to establish that *EST2*, *EST3* and *EST4* defined three genes that were distinct from each other and from *TLC1* and *EST1* (data not shown; see materials and methods for more details). In addition, the products of six to eight tetrads with four viable spores of crosses were analyzed. In all cases, reduced telomere length and senescence showed 2:2 segregation, with these two phenotypes exhibiting 100% co-segregation, demonstrating that a single recessive nuclear mutation was responsible (data not shown).

The lack of recovery of *tlc1* mutants from this screen was surprising given the fact that a defect in *TLC1* gives rise to the same set of phenotypes as observed in *est* mutant strains (Singer and Gottschling 1994; Figures 2, 3). One possible explanation is that many EMS-induced mutations in a gene whose product is an RNA would be expected to be phenotypically silent. However, the absence of *tlc1* mutations from our collection, combined with the fact that only one *est4* isolate was recovered, suggests that there may be additional *EST* genes yet to be identified.

***est2*, *est3* and *est4* mutants are phenotypically indistinguishable from *est1* and *tlc1* strains:** If the three new *EST* genes play a similar role in telomere replication as *EST1* and *TLC1*, mutations in these new genes should display phenotypes similar to those exhibited by *est1*- $\Delta$  and *tlc1*- $\Delta$  strains. To test

this, several mutants from each new complementation group were back-crossed to wild type and sporulated. Freshly generated spore products were examined over time for their growth phenotype and telomere length, in parallel with a freshly generated *est1-Δ* haploid strain.

5 As shown in Figure 2, new *est* mutant isolates exhibit the same phenotypes as *est1-Δ* and *tlc1-Δ* strains. In panel A, Southern blot of genomic yeast DNA grown successively in culture, probed with a telomere-specific probe (poly d[GT]). Lanes 1, 2, 24 and 25 *EST1<sup>+</sup>TLC1<sup>+</sup>*; lanes 3 - 6, *tlc1-Δ::LEU2*; lanes 7 - 10, *est1-Δ3::HIS3*; lanes 11 - 14, *est2-1*; lanes 15 - 18, *est3-1*; lanes 19 - 23, *est4-1*. For the *tlc1*, *est1*, *est2* and *est3* strains, four successive sub-cultures, representing a total of ~65 - 70 generations of growth, are shown; due to the slightly weaker phenotype of *est4-1*, a fifth sub-culture (corresponding to ~13 - 15 additional generations of growth) is included. Lanes 1 and 2 and lanes 24 and 25 show the first and fourth sub-culture, respectively, of two *EST1<sup>+</sup>TLC1<sup>+</sup>* strains handled in parallel. The bracket indicates a telomeric band that represents approximately 2/3 of the telomeres in this strain (those that are Y'-containing); arrows indicate restriction fragments corresponding to individual non-Y'-containing telomeres.

20 Panel B shows the viability of *est1-Δ3::HIS3*, *tlc1-Δ::LEU2*, *est2-1*, *est3-1* and *est4-1*, shown as three successive streak-outs on YEPD plates, differing from each other by about 25 generations of growth; for *est4-1*, one additional successive streak-out is shown.

25 Figure 2A shows the telomere length decline that a representative mutant from each of the new complementation groups displayed over time, as compared to that displayed by *est1-Δ* and *tlc1-Δ* strains. Strains were grown for ~80 generations, with DNA prepared for Southern analysis every ~15 generations. Telomere length in each mutant declined with time, and did so to the same degree as observed in *est1-Δ* and *tlc1-Δ* strains.

30 In parallel with the analysis of telomere length, we also analyzed the growth and chromosome stability over time in these new mutants. Freshly dissected mutant haploids strains were streaked to give single colonies three successive times on YEPD plates, in parallel with freshly generated *est1-Δ* and *tlc1-Δ* haploid strains. Each of the new *est* mutants showed a dramatic

decline in viability late in their outgrowth. The growth phenotype for each of the new *est2* and *est3* alleles was qualitatively indistinguishable from that displayed by null mutations in *EST1* and *TLC1* (Figure 2B and data not shown). The *est4-1* mutant also showed a clear senescence phenotype but in this case, senescence was delayed in comparison to the other *est* mutants, possibly because the single *est4* allele may be somewhat leaky. In parallel with the senescence phenotype, each new *est* mutant also showed a striking increase in the frequency of chromosome loss (data not shown); the appearance of this increased chromosome instability was delayed, similar to the delay previously exhibited by *est1-Δ* strains (Lundblad and Szostak 1989).

**New *est* mutants utilize the same alternative pathway for telomere maintenance as *est1-Δ* or *tlc1-Δ* strains:** Previous analysis has shown that late in the outgrowth of *est1* mutant cultures, a small proportion of cells are able to escape the lethal consequences of the absence of *EST1* function. These *est1-Δ* survivors arise as a result of an bypass pathway for telomere maintenance that is activated in late *est1* cultures (Lundblad and Blackburn 1993). Activation of this alternative pathway occurs as the result of a global amplification and rearrangement of both telomeric G-rich repeats and sub-telomeric regions. This amplification can be quite substantial: in some survivors, the amount of telomeric d(G<sub>1-3</sub>T) repeats is increased by as much as 40 fold, such that 4% of the genome consists of telomeric DNA (V.L., unpublished data). As a consequence of this genomic reorganization, telomere function is restored and the survivors have re-gained a normal or near-normal growth phenotype. This pathway requires the *RAD52* gene, which mediates the majority of homologous recombination events in yeast; in the absence of *RAD52* gene function, *est1 rad52* strains cannot generate late-culture survivors and instead die completely after ~40 to ~60 generations (Lundblad and Blackburn 1993). Consistent with the other similarities between *EST1* and *TLC1*, a *tlc1-Δ* strain also exhibits alterations of telomeric and sub-telomeric DNA (Singer and Gottschling 1994) which is *RAD52*-dependent (Figure 3). This alternative pathway has not been demonstrated to arise in other, non-senescent, telomere replication defective strains of *S. cerevisiae*.

To test whether *est2*, *est3* and *est4* strains were also capable of participating in this process, two to three isolates for each mutant were

grown in culture for 120 to 150 generations and plated for single colonies. Several single colonies from each culture were analyzed for growth characteristics and telomere structure.

As shown in Figure 3, new *est* mutants show the *RAD52*-dependent alternative pathway for telomere maintenance. Panel A shows a Southern blot of genomic yeast DNA. Lanes 1 and 11, a mixture of  $\lambda$  *Hind*III and a single d(G<sub>1,3</sub>T)-containing 4.0 kb fragment detected by the probe; lanes 2 and 10, *EST1*<sup>+</sup>*TLC1*<sup>+</sup>; lanes 3 and 9, early *tlc1-Δ::LEU2*; lanes 4 - 8, survivors isolated from *tlc1-Δ::LEU2*, *est1-Δ3::HIS3*, *est2-1*, *est3-1* and *est4-1*, respectively. A shorter exposure of the region indicated in brackets is shown below, to demonstrate the degree of amplification of Y'-containing sub-telomeric bands (indicated by brackets) that occurs in survivor strains (Lundblad and Blackburn 1993). Note that the probe detects the yeast d(G<sub>1,3</sub>T) telomeric repeats, indicating that both Y' and d(G<sub>1,3</sub>T) sequences are highly amplified in survivors.

Panel B shows the viability of *EST1*<sup>+</sup> *rad52::LEU2*; *tlc1-Δ::LEU2* *rad52::LEU2*; *est1-Δ3::HIS3* *rad52::LEU2*; *est2-1* *rad52::LEU2*; *est3-1* *rad52::LEU2*; and *est4-1* *rad52::LEU2*, shown as two successive streak-outs.

Figure 3A shows the Southern blot of one such survivor from each of the five *tlc1* or *est* strains, probed with poly d(GT); all displayed the same type of telomere rearrangement originally observed in *est1* mutants, characterized by extensive amplification of both d(G<sub>1,3</sub>T) telomeric DNA and Y' sub-telomeric repeats (Lundblad and Blackburn 1993). In addition, each of these *est2*, *est3* and *est4* survivors had now acquired a growth phenotype that closely approximated that of wild type (data not shown), similar to that previously observed for *est1* survivors. The appearance of survivors was not specific to the particular *est* alleles used in Figure 3, as all 19 of the *est* mutants isolated in this mutant screen which displayed a senescence phenotype gave rise to survivors (data not shown).

To test whether the process of generating survivors from *est2*, *est3* and *est4* mutants was also *RAD52*-dependent, each mutant was crossed to a *rad52::LEU2* strain. The resulting diploids were sporulated and dissected, and *est* and *est rad52* haploid strains were analyzed in parallel. Figure 3B shows that the presence of a *rad52* mutation conferred lethality on each

strain after approximately 40 to 50 generations. In addition, the degree of enhancement of the senescence phenotype in the presence of a *rad52* mutation was the same in *est1*, *est2*, *est3* and *tlc1* mutants, providing another point of similarity between mutations in these different genes. Consistent with the somewhat delayed senescence phenotype exhibited by the *est4-1* mutant in a *RAD52<sup>+</sup>* strain (Figure 2B), the appearance of lethality in an *est4-1 rad52::LEU2* strain was slightly delayed (Figure 3B), relative to the other *est rad52* and *tlc1 rad52* mutant strains.

***TLC1* and the four *EST* genes function in a single pathway for telomere replication:** The above comparison demonstrated that strains carrying mutations in the *EST2*, *EST3* and *EST4* genes are phenotypically similar to *est1* and *tlc1* strains; in fact, many of the new *est* isolates are indistinguishable from *est1-Δ* and *tlc1-Δ* strains, suggesting that these alleles may also be null mutations. This similarity in phenotype argues that the new *EST* genes function in the same genetic pathway for telomere replication as originally defined by *EST1*. The alternative possibility is that these five genes operate in more than one pathway, each required to form a functional telomere. These two possibilities can be distinguished by examining the behavior of strains which have multiple mutant combinations. The prediction is that if all five genes function in a single pathway necessary to replicate telomeres, multiply mutant strains should show no enhancement of phenotype. This has already been demonstrated for *EST1* and *TLC1* (Virta-Pearlman, *et al.*, submitted), showing that *EST1* functions in the same *in vivo* pathway as defined by a known component of telomerase. If, however, one or more genes function in a separate, additional pathway required for telomere replication, certain multiple mutant combinations might be expected to show a more severe phenotype. The substantial enhancement of the phenotype of *est* mutant strains that occurs in the presence of a *rad52* mutation (Figure 3B), which eliminates the back-up bypass pathway for telomere maintenance discussed above, is an example of the latter possibility.

To distinguish between these two formal possibilities, each new *est* mutant was crossed against *est1-Δ*, *tlc1-Δ* or the other *est* mutant strains to generate diploids heterozygous at several *EST/TLC1* loci. The subsequent diploids were sporulated and dissected to generate wild type, single mutant

and double mutant spore products. Each double mutant strain was analyzed for telomere length, senescence and chromosome loss (Figure 4 and data not shown), in parallel with single mutants and wild type. In every case, no enhancement of phenotype was seen for double mutant combinations.

5        **Figure 4.** Epistasis analysis of *tlc1* and *est* mutants.

A. Southern blot of genomic yeast DNA. Lanes 1, 2, 15, 16 *EST1<sup>+</sup>TLC1<sup>+</sup>* (representing four successive sub-cultures, handled as in Figure 2A); lanes 2 - 14, three lanes each (three successive sub-cultures) of *est1-Δ3::HIS3*; *est1-Δ3::HIS3 est2-1*; *est1-Δ3::HIS3 est3-1*; and *est1-Δ3::HIS3 est4-1*.

10        B. Southern blot of genomic yeast DNA. Lanes 1, 2, 21, 22, *EST1<sup>+</sup>TLC1<sup>+</sup>*; lanes 3 - 20 three lanes each (three successive sub-cultures) of *tlc1-Δ::LEU2*; *tlc1-Δ::LEU2 est2-1*; *tlc1-Δ::LEU2 est3-1*; *tlc1-Δ::LEU2 est4-1*; *est3-1*; *est2-1 est3-1*.

15        C. Viability of double mutants shown as two successive streak-outs. Top: (counterclockwise from upper left) wild type; *est1-Δ3::HIS3*; *est1-Δ3::HIS3 est2-1*; *est1-Δ3::HIS3 est3-1*; *est1-Δ3::HIS3 est4-1*; *est2-1 est3-1*; Bottom: wild type; *tlc1-Δ::LEU2*; *tlc1-Δ::LEU2 est1-Δ3::HIS3*; *tlc1-Δ::LEU2 est2-1*; *tlc1-Δ::LEU2 est3-1*; *tlc1-Δ::LEU2 est4-1*.

20        Figures 4A and 4B show telomere length for a representative set of double mutant strains compared with the appropriate single mutant strains; in each strain, telomere length declined over time to the same degree. Similarly, as shown in Figure 4C, there was no alteration in the senescence phenotype in any of the double mutant strains, relative to single mutants. This is in striking contrast to the severe enhancement of senescence that  
25        occurred as a result of the introduction of a *rad52* mutation into any of the *est* mutant strains (Figure 3B and 5C). These pairwise combinations argue that each of the new *EST* genes function in the same pathway for telomere replication as defined by *EST1* and *TLC1*.

30        The analysis of various double mutant combinations and their haploid counterparts revealed an unexpected observation, which was that the senescence and telomere shortening phenotypes of single mutant strains was somewhat enhanced when these haploids were derived from a diploid parent that was multiply heterozygous for genes in the *EST/TLC1* pathway. For example, the timecourse of the senescence phenotype of a haploid strain



carrying only the *tlc1-Δ* mutation was accelerated when the *tlc1-Δ* strain was derived from DVL132 (*TLC1<sup>+</sup>/tlc1-Δ EST1<sup>+</sup>/est1-Δ*), compared to the same isogenic haploid *tlc1-Δ* strain derived from DVL131 (*TLC1<sup>+</sup>/tlc1-Δ EST1<sup>+</sup>/EST1<sup>+</sup>*); (compare the *tlc1-Δ* strains in Figure 2B and 4C). This additive haplo-insufficiency was not specific for the *EST1/TLC1* combination and was observed for every possible combination of the five genes in this pathway. Consistent with the accelerated phenotype observed in haploids from these diploids, telomere length was slightly shorter in multiply heterozygous diploids as compared to single heterozygote (D.K.M., unpublished data). Note that the conclusions drawn from the data presented in Figure 4, that additive combinations of different mutations do not show enhancement of phenotype, were made from comparisons between sets of double and single mutant strains derived from the same diploid parent; comparisons to the single mutant haploids in Figure 2 are not valid. Although we do not understand the molecular basis for this phenomenon, it suggests that alterations in gene dosage may be disrupting a complex or a set of interacting complexes.

**The *EST2* gene encodes a novel, highly basic protein:** The wild type *EST2* gene was cloned by complementation of the senescence phenotype of *est2-1*, from both high and low copy genomic libraries (see Materials and Methods for more details). Three independent genomic clones with overlapping inserts were identified which complemented both the growth and telomere length phenotypes of the *est2-1* mutation; no high copy suppressors were identified. Sub-cloning identified a 4.4 kb genomic segment common to all three inserts which was capable of complementing *est2-1*. This fragment was sequenced and also subjected to mutagenesis with the transposable element  $\gamma\delta$  (Strathmann, *et al.*, 1991), in order to identify the genetic boundaries of the *EST2* gene.

**Figure 5. Cloning of the *EST2* gene.**

A. Insertional mutagenesis of the 4.4 kb genomic clone, with the position of the *EST2* open reading frame indicated. Each of the 13  $\gamma\delta$  insertions were assayed for complementation of the telomere shortening and senescence phenotypes of an *est2-1* mutant strain; insertions which fully complement the *Est<sup>-</sup>* phenotype are indicated as an open triangle above the rectangular box

representing the DNA, and failure to complement is indicated by a solid triangle below the representation of the DNA. One insertion, 74 bp upstream of the initiating AUG, exhibited an intermediate phenotype, in that the senescence phenotype was complemented but a telomere shortening phenotype was observed (data not shown). The positions of five small ORFs and a poly-A tract in the promoter region of *EST2* are also indicated.

B. The sequence of the 884 amino acid *EST2* open reading frame.

C. Comparison of the viability of *est1-Δ*, *est2-Δ* and *est1-Δ est2-Δ* strains (both *RAD52* and *rad52* versions), shown as two successive streak-outs.

A total of 13 insertion mutations were isolated, mapped by both PCR and sequence analysis, and assayed for their ability to complement *est2-1* (Figure 5A). These data, combined with the sequence of the 4.4 kb fragment, demonstrated that the complementing activity was due to a 884 amino acid ORF (Figure 5B); no additional ORFs larger than 65 amino acids were found within this sequenced insert.

The promoter region of the *EST2* gene exhibited several notable features found in common with that of *EST1*. First, both genes diverge significantly from the consensus sequence found around the initiating AUG of many yeast genes (Hinnebusch and Liebman 1991; Galibert, *et al.* 1996). In addition, for both *EST1* and *EST2*, the region just upstream of the AUG initiation codon has the potential to encode multiple small ORFs, a feature that is not usually observed in the promoter regions of yeast genes (Cigan and Donahue 1987). In the rare genes where such upstream ORFs have been analyzed, they have been shown to be involved in translational control of gene expression (Hinnebusch 1992). Within 140 bp of the start of the *Est1* protein coding sequence, there are five overlapping ORFs, ranging in size from 3 to 39 codons (Lundblad and Szostak 1989). Similarly, within the first 170 bp of the promoter region of the *EST2* gene, there are five small ORFs, from 4 to 11 codons in length (Figure 5A). Although these small upstream ORFs have not been functionally dissected in detail in either gene, a  $\gamma\delta$  disruption inserted into the cluster of three upstream ORFs was partially defective for *EST2* activity (Figure 5A and data not shown), suggesting that this region may play an important role in expression of *EST2*. One feature of the *EST2* promoter that was not found in *EST1* was a 32 bp long poly (dA) tract just upstream

of the start of the *EST2* coding region. Poly(dA-dT) tracts have previously been implicated in transcriptional activation (Struhl 1986; Lue and Kornberg 1987); the *EST2* poly (dA) tract may also be a promoter element, although it is unusually close to the translational start.

5 To determine the null phenotype and confirm that the *EST2* gene had been cloned, an internal deletion within this ORF was removed and replaced with the *URA3* gene. This construct was introduced into a diploid strain by one-step gene replacement, and the resulting heterozygous strain was sporulated; >80% of the tetrads had four viable spores for each, indicating  
10 that this gene was not essential for immediate viability. The haploid strains from 12 tetrads were further analyzed for senescence and telomere length; for all 12 tetrads, an *est* mutant phenotype co-segregated 100% of the time with the *URA3* marker. The senescence phenotype of *est2-Δ1* was identical to that displayed by an *est1-Δ* strain, both in the presence or absence of *RAD52* gene  
15 function (Figure 5C); furthermore, consistent with the double mutant analysis conducted above with the *est2-1* point mutation, a strain deleted for both genes showed no enhanced phenotype with regard to senescence (Figure 5C) or telomere length (data not shown). In addition, an *est2-Δ1* strain was capable of giving rise to *RAD52*-dependent survivors with a frequency  
20 comparable to that observed for *est1-Δ* and *tlc1-Δ* strains.

The *est2-Δ1* mutation failed to complement the *est2-1* mutation for both telomere length and senescence, and the *est2-1* point mutation was shown by plasmid gap-repair to map within the *EST2* coding region (data not shown), indicating that the correct gene had been cloned.

25 The *EST2* gene encodes a novel 103 kD protein with no similarity to other sequences in the database, nor does it possess any motifs that provide clues as to its function. In particular, there was no sequence similarity between Est2p and either of the two protein sub-units of the Tetrahymena telomerase enzyme (Collins, *et al.* 1995). Like Est1, Est2 is an unusually  
30 basic protein: both proteins have predicted pIs of 10. *TLC1* and the four *EST* genes function in a single pathway for telomere replication: The above comparison demonstrated that strains carrying mutations in the *EST2*, *EST3* and *EST4* genes are phenotypically similar to *est1* and *tlc1* strains; in fact, many of the new *est* isolates are indistinguishable from *est1-Δ* and *tlc1-Δ*

strains, suggesting that these alleles may also be null mutations. This similarity in phenotype argues that the new *EST* genes function in the same genetic pathway for telomere replication as originally defined by the *TLC1* gene.

5

## EXAMPLE 2

### Cloning and sequencing of the wild type *EST3*.

Cloning and sequencing of the wild type *EST3* gene revealed an usual gene structure with a DNA sequence that did not provide immediate clues as to the nature of the *EST3* gene product. The only two ORFs contained within the genetic boundaries of the gene were each less than 100 aa (Figures 7 and 8). This suggested that either: (i) one or both of the ORFs were required for gene function; or (ii) the product of the *EST3* gene was an ORF-less RNA required for telomere replication. To distinguish between these two possibilities, a series of nonsense mutations constructed in either of the two ORFs were shown to eliminate function. In addition, it was shown that several of these nonsense mutations were suppressed in a strain background containing an ochre-suppressing tRNA. These data definitely showed that translation of both ORFs was required for gene function. However, ectopic expression of the two ORFs in trans from separate promoters did not restore gene function. In addition, one of the EMS mutations recovered from our screen mapped to the region between the two ORFs, indicating that genetic information in this region was important for function. Upon more careful re-examination of this region of the *EST3* sequence, we identified a four-nucleotide sequence (CTTA) in common with the +1 frame-shift site found in the yeast retrotransposon elements, Ty1, Ty2 and Ty4, suggesting a similar mechanism for generating the Est3 protein.

To determine whether the *EST3* gene similarly used ribosome frameshifting (abbreviated RF below) to generate a protein product, a mutational analysis of *EST3* was conducted to demonstrate that several components required for Ty RF were also essential for *EST3* RF. To summarize Ty1 ribosome frameshifting, this process depends on an unusual leucine isoacceptor, tRNA<sup>LEU</sup> (UAG), which is capable of recognizing all six leucine codons (Belcourt and Farabaugh 1990). This tRNA can slip from CTT to TTA (thereby slipping to the +1 reading frame) as a consequence of a

translational pause due to low availability of the tRNA decoding the next codon. *EST3* contains the DNA sequence CTT AGT TGA, where AGT is a rarely used codon for serine (and TGA is a nonsense codon). Therefore, by analogy with Ty, our hypothesis was that pausing at the CTT codon, due to the rarely used subsequent codon, allowed slippage to read the codon TTA, thereby changing the reading frame by +1.

To test this, three experimental observations have been made (shown in Figure 11B):

i. changing the CTT codon to another leucine-encoding codon abolishes *EST3* function.

ii. changing from a low (AGT) to high (TCT) usage serine codon abolishes function.

iii. deleting any one nucleotide in the sequence CTTA (to eliminate the need for ribosome frameshifting) results in a functional *EST3* gene.

These genetic results were confirmed by Western analysis demonstrating the production of both the full length Est3 181 amino acid protein product, as well as the truncated 93 aa product which did not undergo frameshifting (Figure 11C).

Figure 11A shows the structure of the *EST3* gene, 11B shows a mutational analysis of the frameshift site and 11C shows the results of a Western analysis of the Gal4 activation domain (lane 1) and the Est3 protein fused to the Gal4 activation domain (lane 2), showing the full length fusion protein and the truncated version terminating after the first ORF.

### EXAMPLE 3

#### Protein Structure.

Having determined the primary structure of the Est2 and Est3 proteins allows the use of the entire proteins and fragments of the proteins to isolate other telomerase-associated proteins. Telomerase-associated proteins are defined as those proteins that are required for the normal *in vivo* functioning of telomerase. These proteins may be associated with the catalytic activity of telomerase or, alternatively, these proteins may be associated with the ability of the telomerase complex to gain access to the telomere.

Fragments of the protein are seen to include any peptide that contains 6 contiguous amino acids or more that are identical to 6 contiguous amino acids of either of the sequences shown in Figures 5,6 and 10. Fragments may be used to generate antibodies. Particularly useful fragments will be those that make up domains of the Est2 or Est3 protein. Domains are defined as portions of the proteins having a discrete tertiary structure and that is maintained in the absence of the remainder of the protein. Such structures can be found by techniques known to those skilled in the art. The protein is partially digested with a protease such as subtilisin, trypsin, chymotrypsin or the like and then subjected to polyacrylamide gel electrophoresis to separate the protein fragments. The fragments can then be transferred to a PVDF membrane and subjected to micro sequencing to determine the amino acid sequence of the N-terminal of the fragments.

#### EXAMPLE 4

##### Expression of telomerase-associated proteins.

Knowledge of the primary structure of the *EST2* and *EST3* gene will permit the over expression of the gene in conventional expression systems. Conventional expression systems are seen to include but are not limited to baculovirus, *E. coli*, vaccinia virus or other equivalent expression systems. For the purposes of this invention an equivalent expression system results in a protein that is either functionally active or antigenically similar to the native protein.

The genes of the present invention may be expressed from the native promoters or may be expressed from heterologous promoters. Promoters may be modified to contain regulatory elements.

In order to facilitate the purification of the recombinantly expressed proteins, the proteins may be expressed in the form of fusion proteins contain heterologous protein sequences. For example, the coding sequence of the *EST2* or *EST3* or fragments thereof may be fused to glutathione-S-transferase (GST). The *EST2* or *EST3* gene or fragments thereof may be fused to other known protein or peptides that are useful to facilitate purification or to permit identification of the fusion protein. Examples of such proteins and peptides are seen to include, but are not limited to, maltose

binding protein, six histidine peptide, epitopes from hemagglutinin epitopes from *c-myc* and any other protein or peptide known in the art for these purposes. Figures 12 and 13 show plasmid maps of specific constructs illustrating the inclusion of a *c-myc* tag at the N-terminal as well as at an internal restriction site. The same construct may contain more than one heterologous protein sequence. When more than one heterologous sequence is included in a construct the heterologous sequences may be the same or different and may be juxtaposed or may be separated by sequences derived from the telomerase-associated protein.

The Est2 or Est3 protein or fragments thereof may be fused to the heterologous proteins at the N-terminal or the C-terminal. When small peptide fusions are made the peptide may be fused at the N-terminal or the C-terminal or the peptide may be fused to the interior of the Est2 or Est3 protein or fragment thereof. In preferred embodiments the heterologous peptide is the hemagglutinin epitope or the *c-myc* epitope and the epitope is fused at the N-terminal, C-terminal, and at locations internal to the Est2 or Est3 protein or fragment thereof. When the epitope is fused internal to the Est2 or Est3 protein or fragment thereof, the location of the epitope will be selected so as not to interfere with the normal functioning of the protein or fragment thereof.

The fusion proteins may be prepared such that the heterologous portion may be cleaved from the fusion protein by the action of a proteolytic enzyme. That is to say, a recognition site for a proteolytic enzyme may be incorporated between the heterologous portion of the fusion protein and the portion derived from the Est2 or Est3 protein or fragment thereof. After purification of the fusion protein, the fusion protein may be subjected to proteolysis in order to remove the heterologous portion of the fusion protein.

The preparation of fusion proteins, their expression, and purification of the resultant fusion proteins may be accomplished by techniques well known to those skilled in the art. Examples of such techniques can be found in Sambrook, *et al.*, Molecular Cloning, 2nd edition and Ausubel, *et al.*, current protocols in Molecular Biology. The disclosure of each of these publications is specifically incorporated herein by reference.

DNA constructs corresponding to the protein sequences shown in Figure 10A-10F were prepared. The DNA sequence corresponding to the wild type Est3 protein was inserted into a plasmid containing the Gal4 activation domain coding sequence fused in frame to the coding sequence for the HA epitope. Expression was driven by the ADH promoter. The plasmids were transfected into yeast strain AVL78 and 1.5 ml cultures were grown to mid-log phase. Cells were pelleted and resuspended in 30  $\mu$ l SDS gel loading buffer (125 mM Tris pH 6.8, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.001% bromophenol blue), incubated at 95 °C for 10 minutes. The samples were clarified by centrifugation at 10,000 rpm in a microfuge and 1-10  $\mu$ l samples were separated by SDS-PAGE on a 12% acrylamide gel. The size of the samples was adjusted to account for varying expression levels of the constructs. The proteins were transferred to nitrocellulose and probed with an anti-HA antibody. The proteins were visualized using horseradish peroxidase conjugated goat anti-mouse antibody and Enhanced ChemiLuminescence (Amersham). Figure 15B lane 1 shows the background plasmid expressing the Gal4-activation domain fused to the HA epitope. Lanes 2 and 7 show the expression of the sequence shown in Figure 10A in which the wild type EST3 gene fused in frame to the Gal4 activation domain-HA construct. Two proteins are expressed corresponding to the non-frameshifted product of 93 amino acids (lower band) and a full length, frameshifted product of 181 amino acids (upper band). Lane 3 shows the expression of a deletion construct having the sequence depicted in Figure 10C in which amino acids 34-164 of the wild type full length protein have been deleted. Lane 4 shows the expression of a truncation construct having the sequence shown in Figure 10E in which tyrosine 35 has been mutated to a stop codon. Lane 5 shows the expression of a truncation construct in which penylalanine 103 has been mutated to a stop codon. Lane 6 shows the expression of a frame shifted construct that can only express the full length 181 amino acid Est3 protein.

#### EXAMPLE 5

##### A new screen in yeast to identify additional telomerase-associate proteins.

This Example proposes a new approach to recover additional genes required for telomere function in yeast. We describe a new mutant screen



which relies on a strain background that is sensitized to the absence of telomerase function. The method described herein is technically much less complex than our previous screen for *EST* genes and allows as many as 10<sup>6</sup> colonies to be screened for *est*-like defects. This method relies upon the observation that a *cdc13-1<sup>ts</sup> tlc1-Δ* strain has two synthetic phenotypes: the maximum permissive temperature of a senescence phenotype as seen in Figure 14. This observation is not unique to the *cdc13-1<sup>ts</sup> tlc1-Δ* strain, in that our unpublished data has shown that *cdc13-1<sup>ts</sup>* strains deleted for either *EST1*, *EST2* or *EST3* also exhibit this behavior. This indicates that this is a general consequence of the loss of the *EST/TLC1* pathway in a *cdc13-1<sup>ts</sup>* background. Therefore, the enhanced temperature sensitivity may be used as the basis for screening for additional mutations which, when in the presence of a *cdc13-1<sup>ts</sup>* mutation, gives the same phenotype.

The procedure employs a plasmid-shuffle strategy, starting with a *cdc13-1<sup>ts</sup>* strain transfected with a *pCDC13-URA3* plasmid. The presence of the plasmid containing a wild-type allele of the *CDC13* gene compensates for the temperature sensitive mutation in the chromosomal copy of the gene.

After mutagenesis, single colonies will be plated on CM-Ura (complete media minus uracil) plates and incubated at 23°C. This selects for colonies that retain the plasmid. Once full-grown, colonies will be replica-plated to 5-FOA plates and incubated at 26°C. The presence of 5-FOA selects for the absence of the plasmid. As a result of this absence, the only allele of *cdc13* present is the mutant copy. As previously shown in Figure 14, when the temperature sensitive allele of *cdc13* is combined with the presence of mutated gene in the *EST/TLC1* pathway, the result is an exacerbation of the temperature sensitive phenotype. Strains carrying only the temperature sensitive allele of *cdc13* grow at 26 °C whereas strains carrying both the temperature sensitive *cdc13* allele and a second mutation in an *EST/TLC1* pathway gene are not viable at 26 °C. Any isolates that fail to grow on the 5-FOA plates at 26 °C will be recovered from the CM-Ura plates.

This primary set of mutagenized cultures will be tested for growth at 26°C on CM-Ura plates, in the presence of the *pCDC13-URA3* plasmid (to rule out the possibility that the second mutation confers a general temperature sensitive phenotype) and re-tested more carefully for the 5-FOA

phenotype at 26°C versus 23°C (to confirm that it is not due to an inability to lose the plasmid at any temperature or failure to grow on 5-FOA). Those candidates that can lose the plasmid at 23°C will be tested for a potential senescence phenotype in the absence of the *CDC13* plasmid; as shown in Figure 14B, this senescence phenotype should be evident after only one streak-out. Finally, candidates that pass these steps will be examined for alterations in telomere length (in the presence of the *CDC13* plasmid, to allow sufficient growth for Southern blots). Since this procedure involves only plating for single colonies and replica-plating, it is greatly simplified relative to our previous screen.

### EXAMPLE 6

#### Two-hybrid system.

One method of using the *EST2* or *EST3* genes or fragments thereof in order to identify additional telomerase-associated proteins is by use of the two-hybrid system. The two-hybrid system uses the restoration of transcriptional activation to indicate an interaction between two proteins. In this system, a eukaryotic transcriptional activator, generally the yeast GAL4 transcription factor, is divided into two domains, a transcriptional activation domain and a DNA-binding domain. Under normal circumstances the two domains are part of the same protein. However, as long as the two domains can be brought into close contact, a functional transcriptional activator can be assembled.

In use, the *EST2* or *EST3* gene or fragments thereof, may be cloned into a plasmid vector containing the coding sequence for the DNA-binding domain. The gene or fragment may be inserted into the vector in such a fashion as to generate an in-frame fusion protein with the DNA-binding domain. A DNA library will be constructed in a plasmid containing the activation domain. The library will be inserted into the plasmid adjacent to the binding domain coding sequence and will result in a fusion protein between the binding protein and the genes represented in the library. The library may be constructed from random DNA fragments or may be constructed from cDNA fragments.

The two plasmids will be cotransformed into an appropriate yeast strain and cotransformants screened for the expression of a functional GAL4 transcriptional activator. This screen may be accomplished by placing a reporter gene, for example  $\beta$ -galactosidase, under the control of a promoter containing a GAL4 responsive element. When an interaction occurs between the *EST2* or *EST3* protein or fragment thereof and a protein in the library, the two GAL4 functional domains will be brought into close proximity. This will result in the restoration of a functional transcriptional activator and expression of the reporter gene. The colonies will be screened for the expression of the reporter gene and colonies that express the reporter gene will be isolated. The plasmid containing the activation domain library fusion protein is recovered and the sequence of the library protein is determined.

The materials necessary to perform this two-hybrid screening system are commercially available, for example from Clontech.

Figure 10 shows the primary structure of a number of GAL4 activation domain fusions prepared with the *EST3* gene. Figure 10A shows a GAL4 activation domain - HA fusion expressing both the first open reading frame and the entire frame shifted gene product. Figure 10B shows the GAL4-HA fusion with the frame shift corrected *EST3*. Figure 10C shows a deletion derivative truncated after the first 50 amino acids of the first open reading frame. Figure 10D shows truncation mutant where phenylalanine 103 was converted to a stop codon. Figure 10E shows a GAL4-HA fusion protein where tyrosine 35 was converted to stop mutation. Figure 10F shows an internal proteolytic fragment of approximately 70 amino acids. Figure 10G shows an *EST3* gene tagged at the C-terminal with three HA epitopes. Figure 10H shows a construct prepared for baculovirus expression wherein the Est3 protein has been tagged on the C-terminal with six histidine amino acids.

Other fusion proteins that have been prepared include a GST-Est2 fusion protein.

**EXAMPLE 7**Production of antibodies.

After expression and purification of *Est2* and *Est3* proteins or fragments thereof the proteins or fragments may be used to generate antibodies specific to the proteins or fragments thereof. The antibodies may be polyclonal or monoclonal. The production of antibodies will be accomplished using conventional techniques well known to those of skill in the art. The techniques used can be found in a variety of references, for example, Harlow and Lane, Antibodies, A Laboratory Manual, the disclosure of which is specifically incorporated herein by reference.

In brief the protein or fragment thereof may be purified from native sources or, alternatively, may be purified from a heterologous over expressing host. When fragments are used, the fragments may be prepared from purified protein by treatment with protease enzymes or alternatively, the fragments may be prepared synthetically using solid phase synthesis technology well known to those of skill in the art. The purified protein or fragment may be mixed with an adjuvant, for example, Freund's complete adjuvant or Freund's incomplete adjuvant or other synthetic adjuvants known to those of skill in the art. The adjuvant/protein mixture may be injected into a laboratory animal in order to produce an immune response. The immune response may be boosted by the injection of additional protein material with or without adjuvant. Injection schedules and regimens are well known to those skilled in the art. When antibodies specific to peptides are being made, the peptide may be conjugated to a carrier molecule such as keyhole limpet hemocyanin (KLH), or any other carrier protein known in the art.

**EXAMPLE 8**Identification of peptides or small molecules that inhibit telomerase function.

The current approach to the development of anti-telomerase drugs is to search for compounds that inhibit enzyme activity, a tactic which is being pursued in a number of both academic and commercial laboratories. This example, however, focuses on an alternative and innovative means of telomerase inhibition, by inhibiting not the enzyme itself but by preventing

access of the enzyme to the telomere. This is a new concept for telomerase inhibition, based on recent research in our laboratory which has led to the identification and characterization of a telomere binding protein that is required for telomerase access to the chromosome terminus. Parallel  
5 development of inhibitors of both enzyme activity and enzyme access may provide the opportunity for a more powerful, combined anti-telomerase cancer therapy, based on the example of the increased efficacy of the combined use of protease and reverse transcriptase inhibitors in the treatment of HIV infection.

10 Characterization of the yeast *CDC13* gene (identified in our est mutant screen) has shown that it has a dual role in telomere function. The *CDC13* gene is an essential yeast gene that is required for maintaining telomere integrity, as first suggested by a study from Lee Hartwell's laboratory showing that a rapid loss of one strand of the telomere occurs in the absence  
15 of *CDC13* function. Recent work from our laboratory has uncovered an additional role for *CDC13* in telomere maintenance. This resulted from the discovery of a novel mutation, called *cdc13-2eSt*, that displayed a phenotype virtually identical to that of a telomerase-minus strain. Furthermore, epistasis analysis between the *cdc13-2eSt* mutation and *tlcl-A* demonstrated  
20 that *cdc13-2eSt* perturbs a function of *CDC13* that is required for the telomerase pathway. In contrast, genetic analysis of the previously isolated conditional lethal allele of *CDC13* (*cdc13-lts*) showed that *CDC13* has a separate essential function that must be maintained in addition to the telomerase-based pathway. We have also shown that purified Cdc13 protein  
25 binds specifically to single-stranded yeast telomeric substrates. Based on these data, we propose that Cdc13 has two distinct functions at the telomere. The first of these roles is to protect the end of the chromosome, which is essential for cell viability. Second, Cdc13 protein regulates telomerase by mediating, either directly or indirectly, access of this enzyme to the  
30 chromosomal terminus, and that this access is now eliminated by the *cdc13-2eSt* mutation. This second role provides the basis for blocking access of the enzyme telomerase to the chromosomal terminus, either by mutation or potentially via the binding of a small molecule or peptide inhibitor to Cdc13p, at a site defined by the *cdc13-2eSt* mutation. This then provides an

alternative means of inhibition of telomerase activity, via the identification of inhibitors that block access of the enzyme to the telomere. We propose to identify the human Cdc13 protein and using it as a tool to identify peptides that bind to this proposed telomerase loading site, via the use of phage-displayed random peptide libraries to identify peptides that will have antagonistic effects on the telomerase-related function of *CDC13*.

Random peptide and small molecule libraries may be screened in a variety of ways known to those of skill in the art. One preferred embodiment will be to screen a phage display library. The technique employs fusion of random peptide sequences to either of two proteins that make up the phage coat of filamentous phage. Multiple rounds of enrichment for phage that exhibit tight binding to the selector protein is achieved via either affinity chromatography or panning.

An alternative method for screening both random peptide libraries and small molecule libraries is to use *in vitro* telomerase assays and test for inhibition of the enzymatic activity of the telomerase. This type of assay can readily be automated using robotic instrumentation to rapidly assay large quantities of samples. The ability to establish suitable enzymatic assays and the analysis of inhibition data are well within the purview of those of ordinary skill in the art.

High complexity libraries will be used. The libraries may incorporate a number of recent modifications such as: monovalent display, that allow selection for higher affinity ligands; structurally constrained libraries; and libraries displaying D-peptide ligands. In addition, more than one library will be screened in order to increase the odds of recovery and to expand the range of peptides recovered.

When screening with the Cdc13 protein, alternate rounds of selection and anti-selection will be used. First a round using wild-type Cdc13 protein will be conducted. This will select for peptides and/or small molecules that are capable of binding to the wild type Cdc13 protein. Following this step, a round of anti-selection with the Cdc13<sup>mut</sup> protein, which contains a mutation that abolishes binding to the telomerase complex, will eliminate those peptides and small molecules that bind to random sites on the Cdc13 protein. Therefore, in the anti-selection step, small molecules, peptides and phage

which do not bind to the Cdc13<sup>ts</sup> protein will be recovered. Each library will be screened two to four times to ensure full coverage.

Peptides and small molecules identified as binding to the Cdc13 protein will be screened for the ability to inhibit telomerase activity and such peptides and small molecules will be useful *in vivo* to prevent telomerase function, thereby preventing unlimited replication potential. When the selection protein is derived from a human telomerase complex, the peptides identified will find use as anti-tumor agents to suppress the unlimited replication of tumor cells. When the selection proteins are derived from pathogenic organisms, the peptides identified by this screen may be used to inhibit the growth of the pathogenic organism.

### EXAMPLE 9

#### Identification of mammalian homologues of the four EST genes.

In this example, methods employing several molecular and/or genetic approaches to use the cloned yeast *EST* genes, as well as the *est*<sup>-</sup> mutant strains, as reagents for obtaining the equivalent human genes are disclosed. One method will be functional complementation. Examples of two human cDNA libraries in yeast expression vectors are the inducible yeast GAL promoter, providing regulated transcription, present on a high copy yeast shuttle vector (Ninomiya-Tsuji, *et al.*, 1991); the second library was constructed using the constitutive yeast *ADH1* promoter and is also present on a high copy yeast vector (Becker, *et al.*, 1991). In addition, two different cDNA libraries (with and without nuclear localization signals) from 24 *K. lactis* behind the *S. cerevisiae* ADH promoter in the pYES2 vector (Pearlman, Becherer and Lundblad, unpublished) have been constructed for use in identifying homologs from more closely related species.

Freshly dissected haploid *est2*<sup>-</sup> *rad52*<sup>-</sup> and *est3*<sup>-</sup> *rad52*<sup>-</sup> strains will be transformed with either of these libraries and healthy-growing transformants identified; the presence of the *rad52* mutation is necessary to prevent the appearance of a bypass pathway that gives rise to healthy growing derivatives (Lundblad and Blackburn, 1993). In the case of the *EST4/CDC13* gene, the libraries will be screened for complementation of (i) the senescence phenotype of *est4* *rad52* , (ii) the ts phenotype of a *cdc13-ts* mutation and (iii) the

lethality of a *est4/cdc13*- (this latter experiment will be performed using a plasmid shuffle type of strategy); complementation of these three different types of mutations may help the ability to recover a complementing clone. A second method to isolate homologs will be by cross-hybridization and degenerate PCR. Based on the results that we have obtained with *EST1*, as well as preliminary results screening a zoo blot of distantly related species with *EST2*, *EST3* and *EST4* probes, it is likely that we will need to collect and sequence a set of 3 to 4 homologs for each EST gene from somewhat related species. This information will be used to look for amino acid sequence patterns that are signatures for the Est3 protein. These amino acid signature sequences will be used to search the protein sequence data bases using a pattern recognition search program (Smith and Smith, 1990) for proteins which contain these motifs. Protein sequences identified by this procedure will be analyzed for whether there are additional sequence features suggesting that we have identified an Est3 candidate. Any promising candidates will be analyzed in more detail as described below.

These amino acid signature sequences can also be used to design degenerate PCR primers against highly conserved regions to be used for a substantial cross-species jump. If more than two highly conserved regions in a given EST gene are identified, nested degenerate PCR reactions will be performed. PCR products will be size-selected, cloned and sequenced, and sequence information will be evaluated to determine whether we have identified an *EST* homolog. Promising PCR clones will be used to identify and sequence full length cDNAs from the appropriate library.

The source of the cDNA libraries used will be the two HeLa cDNA libraries described above. Since both are in vectors that allow expression of the cloned human gene in yeast, this has the advantage that candidate genes can be rapidly screened for whether they confer a phenotype in yeast as discussed below.

Several types of experiments will be used to demonstrate whether the correct gene has been isolated. Sequence comparisons will be used if the gene has been identified by a PCR jump from a homolog database. Second, each candidate cDNA will be tested for whether it exerts a genetic phenotype in yeast. Each cDNA will be checked for complementation of an *est3* mutation.



If there is no evidence for complementation, each cDNA will be subsequently tested for whether it can confer a dominant effect on telomere replication in wild type yeast; in other words, over-expression of such a cDNA may inhibit yeast telomere replication by interfering with the formation of a necessary yeast complex (such as telomerase).

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Table 1  
Yeast strains

5	Strain	Genotype <sup>a</sup>	Source
	YPH275	<i>MATa/MATa CF-SUP11-TRP1</i>	P. Hieter
	TVL120	<i>MATa/MATa EST1/est1-Δ3::HIS3 CF-SUP11-TRP1</i>	This study
10	TVL140	<i>MATa/MATa EST1/est1-Δ3::HIS3 RAD52/rad52::LEU2</i> <i>CF-SUP11-TRP1</i>	This study
	TVL228	<i>MATa/MATa EST1/est1-Δ3::HIS3 RAD52/rad52::LEU2</i> <i>EST2/est2-Δ1::URA3 CF-SUP11-TRP1</i>	This study
	DVL131	<i>MATa/MATa TLC1/tlc1-Δ::LEU2 EST1/est1-Δ3::HIS3</i> <i>CF-SUP11-TRP1</i>	This study
15	DVL132	<i>MATa/MATa TLC1/tlc1-Δ::LEU2 CF-SUP11-TRP1</i>	This study
	TVL227-1A	<i>MATa CF-SUP11-TRP1 /pVL106<sup>b</sup></i>	This study
	MVL6 <sup>c</sup>	<i>MATa est1-19</i>	This study
20	MVL9	<i>MATa est1-20</i>	This study
	MVL11	<i>MATa est1-21</i>	This study
	MVL12	<i>MATa est1-22</i>	This study
	MVL16	<i>MATa est1-23</i>	This study
	MVL17	<i>MATa est1-24</i>	This study
25	MVL18	<i>MATa est1-25</i>	This study
	MVL19	<i>MATa est1-26</i>	This study
	MVL20	<i>MATa est1-27</i>	This study
	MVL21	<i>MATa est1-28</i>	This study
	MVL22	<i>MATa est1-29</i>	This study
30	MVL23	<i>MATa est1-30</i>	This study
	MVL24	<i>MATa est1-31</i>	This study
	MVL7	<i>MATa est2-1</i>	This study
	MVL8	<i>MATa est2-2</i>	This study
	MVL13	<i>MATa est2-3</i>	This study
35	MVL14	<i>MATa est2-4</i>	This study
	MVL10	<i>MATa est3-1</i>	This study
	MVL15	<i>MATa est3-2</i>	This study
	MVL25	<i>MATa est3-3</i>	This study
	MVL1	<i>MATa est3-4</i>	This study
40	MVL26	<i>MATa est4-1</i>	This study



Table 1 (continued)

<sup>a</sup> All strains were isogenic derivatives of YPH275; the diploids also carry *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ1/trp1-Δ1 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1* and the haploids carry *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*

<sup>b</sup> pVL106 is an *ARS1 LEU2 CEN3* plasmid used in the plasmid linearization assay; see Lundblad and Szostak 1989 for more details.

<sup>c</sup> Although MVL1 - MVL26 are derived from TVL227-1A, during the course of the screen, many mutant isolates lost either CF or pVL106 (or both); therefore, these two elements are not indicated in the genotypes of MVL1 - MVL26.

What is claimed is:

- 1        1.    An isolated protein having at least the sequence shown in Figure 5.
- 1        2.    A peptide having at least six contiguous amino acids identical to six  
2                contiguous amino acids of the protein sequence in Figure 5.
- 1        3.    The peptide according to claim 2, further comprising a GAL4 DNA  
2                binding domain.
- 1        4.    The protein according to claim 2, further comprising a GAL4 activation  
2                domain.
- 1        5.    The peptide according to claim 2, further comprising a peptide epitope.
- 1        6.    The peptide according to claim 5, wherein the epitope is a hemagglutinin  
2                epitope.
- 1        7.    The peptide according to claim 5, wherein the peptide epitope is a *c-myc*  
2                epitope.
- 1        8.    A peptide according to claim 5, wherein the epitope is fused to the N-  
2                terminal of the peptide.
- 1        9.    The peptide according to claim 5, wherein the epitope is fused to the C-  
2                terminal of the peptide.
- 1        10.   The peptide according to claim 5, wherein the epitope is fused to the  
2                interior of the peptide.
- 1        11.   A DNA molecule coding for the protein sequence in Figure 5.

- 1        12. A DNA molecule coding for a peptide having at least six contiguous  
2            amino acids identical to six contiguous amino acids of the protein  
3            sequence of Figure 5.
- 1        13. An isolated protein having at least the sequence shown in Figure 6.
- 1        14. A peptide having at least six contiguous amino acids identical to six  
2            contiguous amino acids of the protein sequence in Figure 6.
- 1        15. The peptide according to claim 14, further comprising a GAL4 DNA  
2            binding domain.
- 1        16. The protein according to claim 14, further comprising a GAL4 activation  
2            domain.
- 1        17. The peptide according to claim 14, further comprising a peptide epitope.
- 1        18. The peptide according to claim 17, wherein the epitope is a  
2            hemagglutinin epitope.
- 1        19. The peptide according to claim 17, wherein the peptide epitope is a *c-*  
2            *myc* epitope.
- 1        20. A peptide according to claim 17, wherein the epitope is fused to the N-  
2            terminal of the peptide.
- 1        21. The peptide according to claim 17, wherein the epitope is fused to the C-  
2            terminal of the peptide.
- 1        22. The peptide according to claim 17, wherein the epitope is fused to the  
2            interior of the peptide.
- 1        23. A DNA molecule coding for the protein sequence in Figure 6.

1 24. A DNA molecule coding for a peptide having at least six contiguous  
2 amino acids identical to six contiguous amino acids of the protein  
3 sequence of Figure 6.

1 25. A method for identifying genes encoding telomerase-associated proteins  
2 comprising the steps of:

3 transfecting a temperature sensitive organism, the organism being  
4 temperature sensitive in a first telomerase-associated gene, with a  
5 plasmid encoding a wild-type allele of the first telomerase-associated  
6 gene in which the organism is temperature sensitive, the plasmid  
7 encoding a selection gene;

8 contacting the organism with a mutagen;

9 growing the mutagenized organism at a permissive temperature  
10 on a media containing a substance that selects for the presence of the  
11 plasmid;

12 growing the organism at a temperature higher than a permissive  
13 temperature on a media containing a substance that selects against the  
14 presence of the plasmid; and

15 identifying those organisms that fail to grow at a temperature  
16 higher than a permissive temperature;

17 identifying a mutated gene, other than the first telomerase-  
18 associated gene, in the organism that fails to grow at a higher than  
19 permissive temperature, wherein the mutated gene is a telomerase-associated  
20 gene.

1 26. A method according to claim 25, wherein the organism is yeast and the  
2 gene in which the yeast is temperature sensitive is *CDC13*.

1 27. An antibody that binds the protein of claim 1.

1 28. An antibody according to claim 27, wherein said antibody is a polyclonal  
2 antibody.

- 1 29. An antibody according to claim 27, wherein said antibody is a  
2 monoclonal antibody.
- 1 30. An antibody capable of binding the protein of claim 13.
- 1 31. An antibody according to claim 30, wherein said antibody is polyclonal  
2 antibody.
- 1 32. An antibody according to claim 30, wherein said antibody is a  
2 monoclonal antibody.
- 1 33. An antibody that binds to the peptide of claim 2.
- 1 34. An antibody according to claim 33, wherein said antibody is a polyclonal  
2 antibody.
- 1 35. An antibody according to claim 33, wherein said antibody is a  
2 monoclonal antibody.
- 1 36. An antibody that binds to the peptide of claim 14.
- 1 37. An antibody according to claim 36, wherein said antibody is a polyclonal  
2 antibody.
- 1 38. An antibody according to claim 35, wherein said antibody is a  
2 monoclonal antibody.
- 1 39. A method for isolating telomerase-inhibiting compounds, comprising the  
2 steps of:  
3 contacting a library of compounds with a selection protein; and  
4 isolating a compound that binds to the selection protein, wherein  
5 the selection protein is a telomerase-associated protein.

- 1       40. A method according to claim 39, wherein the selection protein is encoded  
2       by an *EST1* gene.
- 1       41. A method according to claim 39, wherein the protein is encoded by an  
2       *EST2* gene.
- 1       42. A method according to claim 39, wherein the selection protein is encoded  
2       by *EST3* gene.
- 1       43. A method according to claim 39, wherein the selection protein is encoded  
2       by a *CDC13* gene.
- 1       44. A method according to claim 39, wherein the compounds are selected  
2       from the group consisting of peptides and small molecules.
- 1       45. A method for identifying homologs to telomerase-associated proteins,  
2       comprising the steps of:  
3             identifying telomerase-associated proteins in two or more  
4       organisms;  
5             identifying at least one motif that is conserved in all of the  
6       organisms; and  
7             searching a database for proteins containing the conserved motifs  
8       wherein proteins having a conserved motif are telomerase-associated  
9       proteins.

Fig. 1

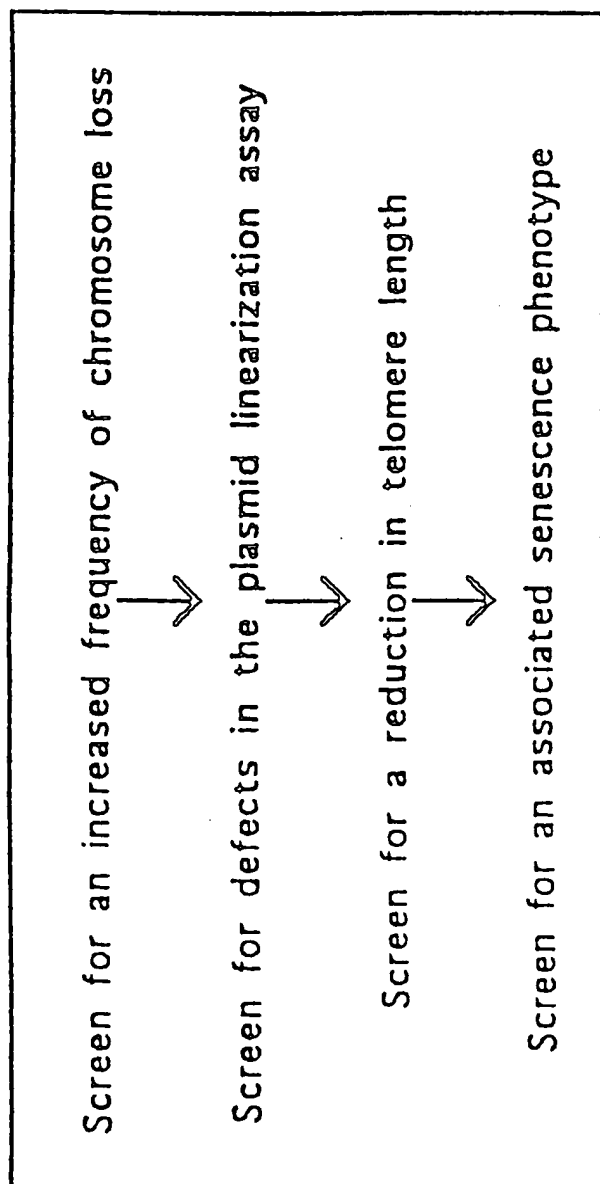
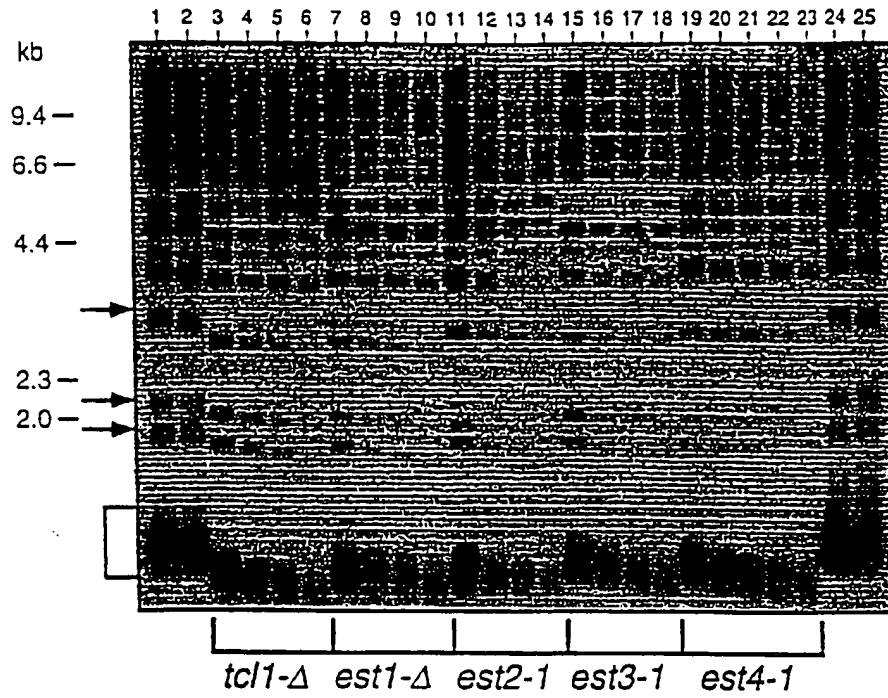


Fig. 2

A



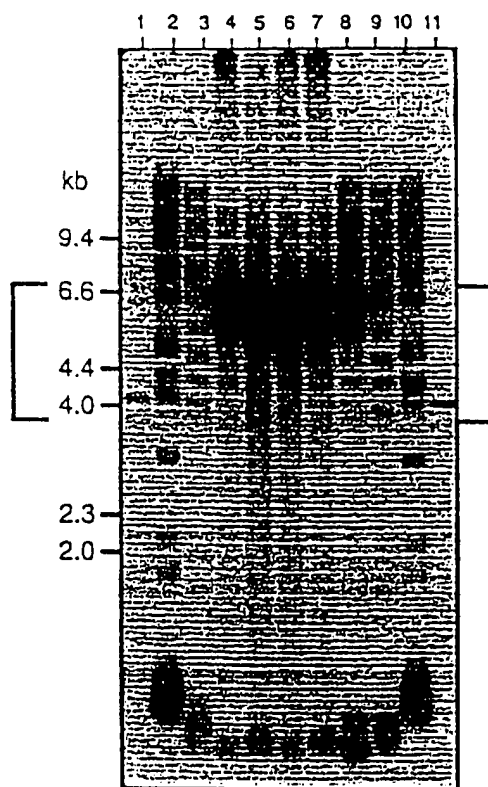
B



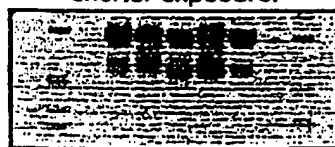


Fig. 3

A



shorter exposure:



survivor strains

*rad52*

*luc1-Δ*  
*rad52*

*est1-Δ*  
*rad52*

*est2-1*  
*rad52*

*est3-1*  
*rad52*

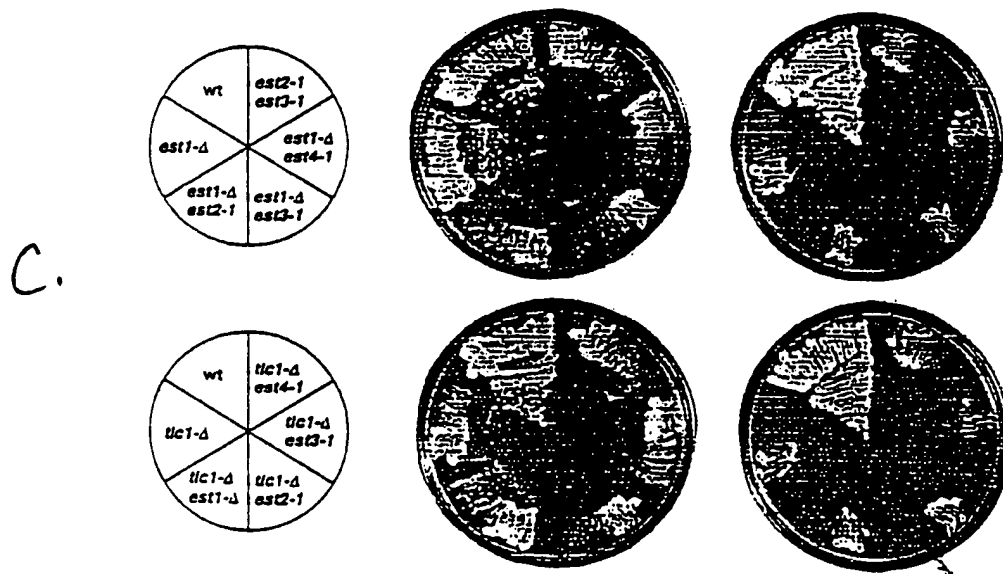
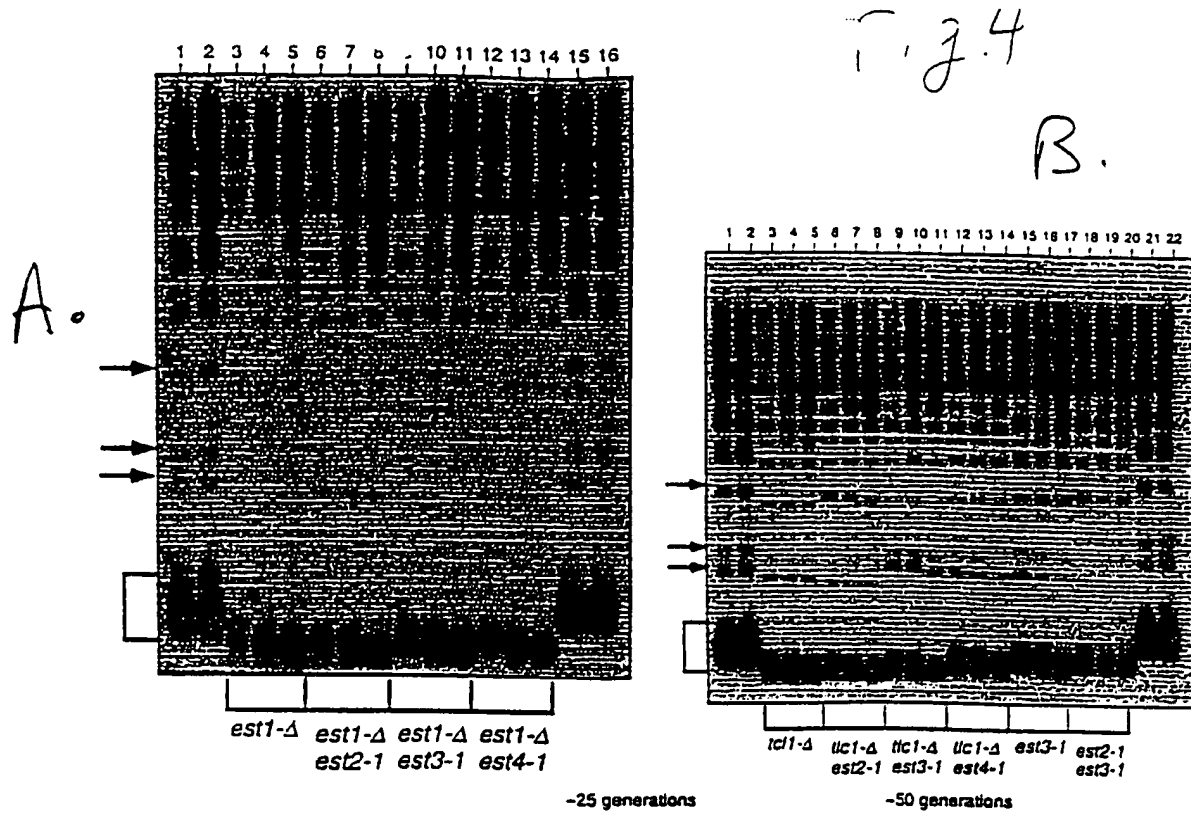
*est4-1*  
*rad52*

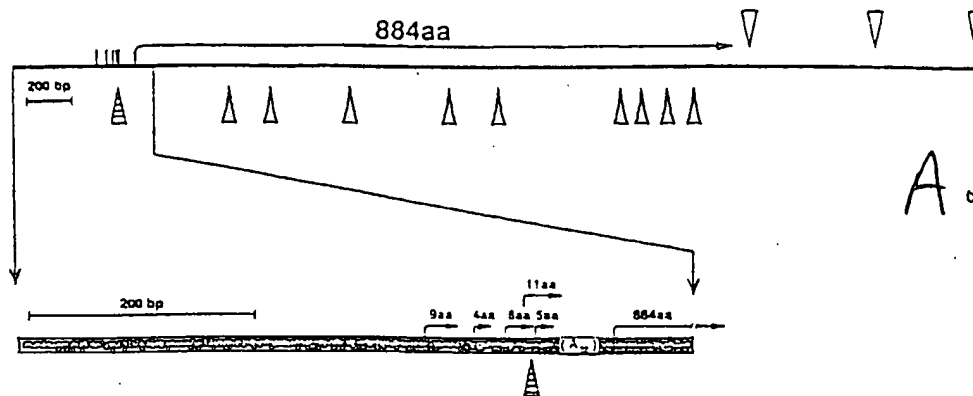
~25  
generations

~50  
generations



B





1 MKILFEFIQD RLDIDLQTN S TYKENLRCGE FNGLDEILTT CFALPNSRKI  
 51 ALPCLPGDLS EKAVIDHCII YLLTGELYNN VLTFGYKIAR NEDVNSSLFC  
 101 ESANVNVTL L KGAANKMFHS LVGTIAFVDL LINYTVIQFN GQFFTQIVGN  
 151 RCNEPHELPPK WVORSSSSSA TAAQIKQLTE PVTNRQFLHK LNINSSSFFP  
 201 YSKILPSSSS IKKLTDLREA IFPTNLVKIP QRLKVRINLT LQKLLRHKR  
 251 LNYVSILNSI CPPLEGTVD LSHLSRQSPK ERVLRPIIVI LQKLLPQEMF  
 301 GSKNKGKII KNLNLLSLP LNGYLPFDSL LKKLRKDFR WLFISDIWFT  
 351 KHNFNENQL AICFISWLR QLIPKIIQT FYCTEISSTV TIVYFRHOTW  
 401 NKLITPPIVE YFKTYLVENN VCRNHSYTL SNFNBSKMRI IPKKSNEFR  
 451 IIAIPCRGAD EEESTIYKEN HKNAIQPTQK ILEYLRNKR TPSTRIYSPT  
 501 QIADRIKEFK QRLLEKFN NV LPELYPMKFD VKSCYDSIPR MECHRIKDA  
 551 LKNENGFFVR SQYFNTNTG VLKLFNVVNA SRVPKPYELY IDNVRTVHLS  
 601 NQDVINVVEM ZIFKTALWE DKCYIREDGL FQGSSLSAPI VDLVYDDLLE  
 651 FYSEFKASPS QDTLILKLAD DFLIISTDQO QVINIKKLAM GGFQKYNKA  
 701 NRDKILAVSS QSDDDTVIQF CAMBIFVKEL EVWKHSSTMN NFHIRSKSSK  
 751 GIPRSLIALF NTRISYKTID TNLNSTNTVL MQIDHVVKNI SECYKSAFKD  
 801 LSINVTQNMQ FBSFLQRIE MTVSGCPITK CDPLIEYEV FTLNGFLES  
 851 LSSNTSKFKD NIILLRKEIQ HLQAYIYIYI HIVN

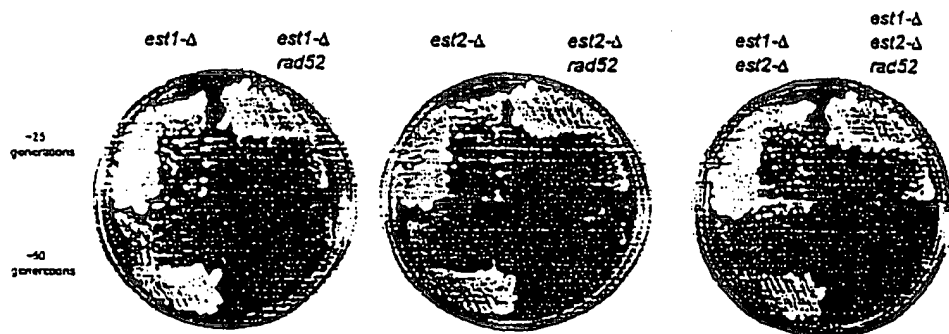


FIG 6

EST3 :

MPKVILESHSKPTDSVFLQPWIKALIEDNSEHDQYHPSGHVIPSLTKQDLALPHMSPTILT  
NPCHFAKITKFYNVCDYKVYASIRDSSHQILVEFSQECVSNFERTHNCRITSETTNCLMII  
GDADLVYVNSRAMSHFKICLSNISSKEIVPVLNVNQATIFDIDQVGSLSLTFPFVYKYL

GGATGATAGTTGGTACTTATTTCAACAAATGATACGTTTTAAACTGCTAGTTATCAACAC  
1 -----+-----+-----+-----+-----+-----+ 60

TTTTCTACCAGATTCTCATTATATTTTAAAAAACATATACTATACAAATAAACGAAATT  
61 -----+-----+-----+-----+-----+-----+ 120

TTCATAGAAACAAATCCGTGATAGTTTAATGGTCAGAATGGGCGCTTGTCGCGTGCCAGA  
121 -----+-----+-----+-----+-----+-----+ 180

TCGGGGTTCAATTCCCCGTCGCGGAGATTTTTTACCATAACTGATGACTACAGTTGGTG  
181 -----+-----+-----+-----+-----+-----+ 240

AAACGGTAGCGAGTAAATATGAAACTAGACAGCAAAAGTTGACCATAGATAATGAATTAA  
241 -----+-----+-----+-----+-----+-----+ 300

AAGCTGTAAATCCTGGGTACAAATCGGGATAACAAGTAAACAATGCCGAAAGTAATTCTG  
301 -----+-----+-----+-----+-----+-----+ 360

M P K V I L -

GAGTCTCATTCAAAGCCAACAGACTCAGTTTTTCTACAACCATGGATAAAGGCATTAATT  
361 -----+-----+-----+-----+-----+-----+ 420

a E S H S K P T D S V F L Q P W I K A L I -

GAAGACAACCTCGGAGCATGATCAATATCATCCCTCTGGCCATGTAATTCCTAGCTTGACC  
421 -----+-----+-----+-----+-----+-----+ 480

a E D N S E H D Q Y H P S G H V I P S L T -

AAGCAGGACTTAGCGCTACCGCATATGAGCCCGACAATTTTAACCAATCCGTGCCATTTC  
481 -----+-----+-----+-----+-----+-----+ 540

a K Q D L A L P H M S P T I L T N P C H F -

FI 6.7  
(cont)

GCCAAAATTACAAAATTTTATAACGTTTGCGACTACAAGGTATACGCATCGATAAGAGAT  
541 -----+-----+-----+-----+-----+-----+-----+ 600  
a A K I T K F Y N V C D Y K V Y A S I R D -  
b \* E I -

TCCTCACACCAAATACCTTAGTTGAGTTTCCCAAGAGTGTGTATCTAATTTTGAAAGGAC  
601 -----+-----+-----+-----+-----+-----+-----+ 660  
a S S H Q I L S \*  
b P H T K Y L V E F S Q E C V S N F E R T -

← this where the ribosome frameshift occurs

TCATAATTGCAGGATCACATCTGAGACGACCAATTGCTTAATGATCATTGGCGATGCTGA  
661 -----+-----+-----+-----+-----+-----+-----+ 720  
b H N C R I T S E T T N C L M I I G D A D -

CTTAGTCTACGTAACAAATTCTCGAGCAATGTCTCACTTCAAAATTTGCCTAAGCAACAT  
721 -----+-----+-----+-----+-----+-----+-----+ 780  
b L V Y V T N S R A M S H F K I C L S N I -

TTCGTCAAAGAAATAGTGCCCGTTCTCAATGTAAACCAGGCCACGATATTTGATATTGA  
781 -----+-----+-----+-----+-----+-----+-----+ 840  
b S S K E I V P V L N V N Q A T I F D I D -

TCAAGTCGGATCGTTAAGTACTTTCCCATTTGTATATAAAATTTTATGACCTTCTGCAGG  
841 -----+-----+-----+-----+-----+-----+-----+ 900  
b Q V G S L S T F P F V Y K Y L \*

CTCGATATTTTATAAGTGTGAGAGAGTTAGAATATCTTCGTTTATCTACATATATAAAGG  
901 -----+-----+-----+-----+-----+-----+-----+ 960

FIG. 7  
(cont)

GAAGGGTTTGGGAATATTTTACCGATAAGTACTCCTCTAGAGAAACAAAAAGGGGGTTATTT  
961 -----+-----+-----+-----+-----+-----+ 1020

AAACTTCATTCTTCTTTAAACTTTTCAGCGACTTCTAAAACCTCCTTTTTGGCGTCATTT  
1021 -----+-----+-----+-----+-----+-----+ 1080

ACACTAACTTCTGGTGATATCTTAACTCTTTTGAATTTTAACTTCCCATCAACAAAAATG  
1081 -----+-----+-----+-----+-----+-----+ 1140

AAATG  
1141 ----- 1145

FIG. 8

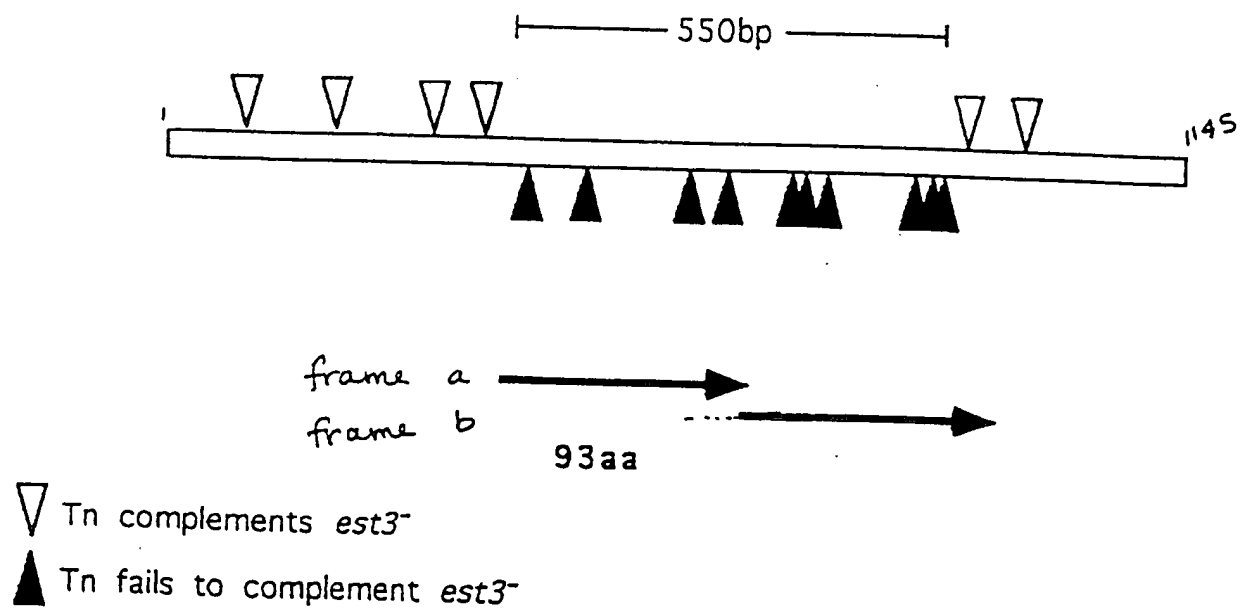
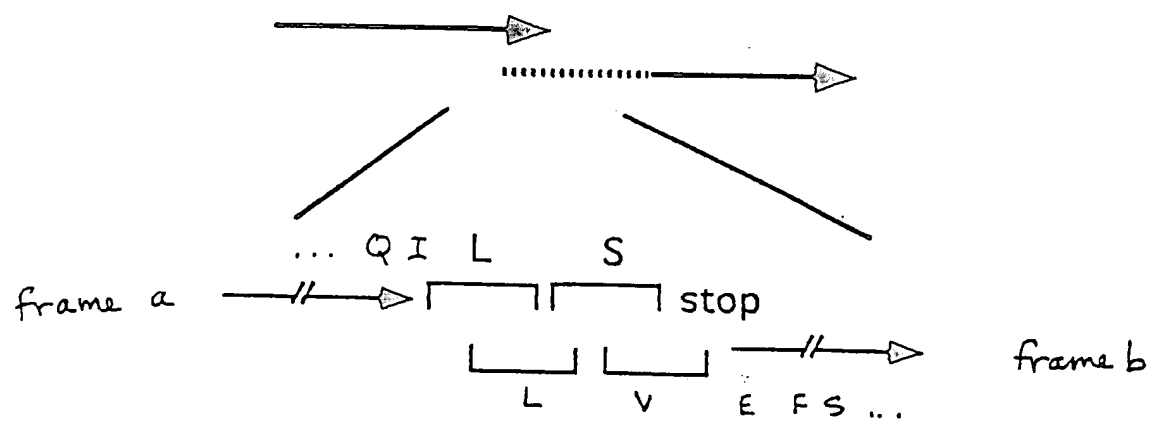




FIG. 9



## Figure 10

A - F are *EST3* genes overexpressed by the ADH promoter and are N-terminally tagged with the Gal4-activating domain and the HA epitope.

A. wild type *EST3*:

(Gal4AD-HA)-MPKVILESHSKPTDSVFLQPWIKALIEDNSEHDQYHPSGHVIPS LTK  
QDLALPHMSPTILTNPCHF AKITKFYNVCDYKVYASIRDSSHQILS

plus

(Gal4AD-HA)-MPKVILESHSKPTDSVFLQPWIKALIEDNSEHDQYHPSGHVIPS LTK  
QDLALPHMSPTILTNPCHF AKITKFYNVCDYKVYASIRDSSHQILVEFSQECVSNFERTHN  
CRITSETTNCLMIIGDADLVYVNSRAMSHFKICLSNISSKEIVPVLNVNQATIFDIDQVGS  
LSTFPFVYKYL

B. frameshift corrected *EST3*:

(Gal4AD-HA)-MPKVILESHSKPTDSVFLQPWIKALIEDNSEHDQYHPSGHVIPS LTK  
QDLALPHMSPTILTNPCHF AKITKFYNVCDYKVYASIRDSSHQILVEFSQECVSNFERTHN  
CRITSETTNCLMIIGDADLVYVNSRAMSHFKICLSNISSKEIVPVLNVNQATIFDIDQVGS  
LSTFPFVYKYL

C. deletion derivative:

(Gal4AD-HA)-MPKVILESHSKPTDSVFLQPWIKALIEDNSEHDH DQVGS LSTFP  
FVYKYL

D. F103->stop mutant

(Gal4AD-HA)-MPKVILESHSKPTDSVFLQPWIKALIEDNSEHDQYHPSGHV  
IPSLTKQDLALPHMSPTILTNPCHF AKITKFYNVCDYKVYASIRDSSHQILVEFSQECVSN

Fig. 10  
(cont)

E. Y35-&gt; stop mutant

(Gal4AD-HA) -MPKVILESHSKPTDSVFLQPWIKALIEDNSEHDQ

F. N-terminal proteolytic fragment: ~70aa (8kD)

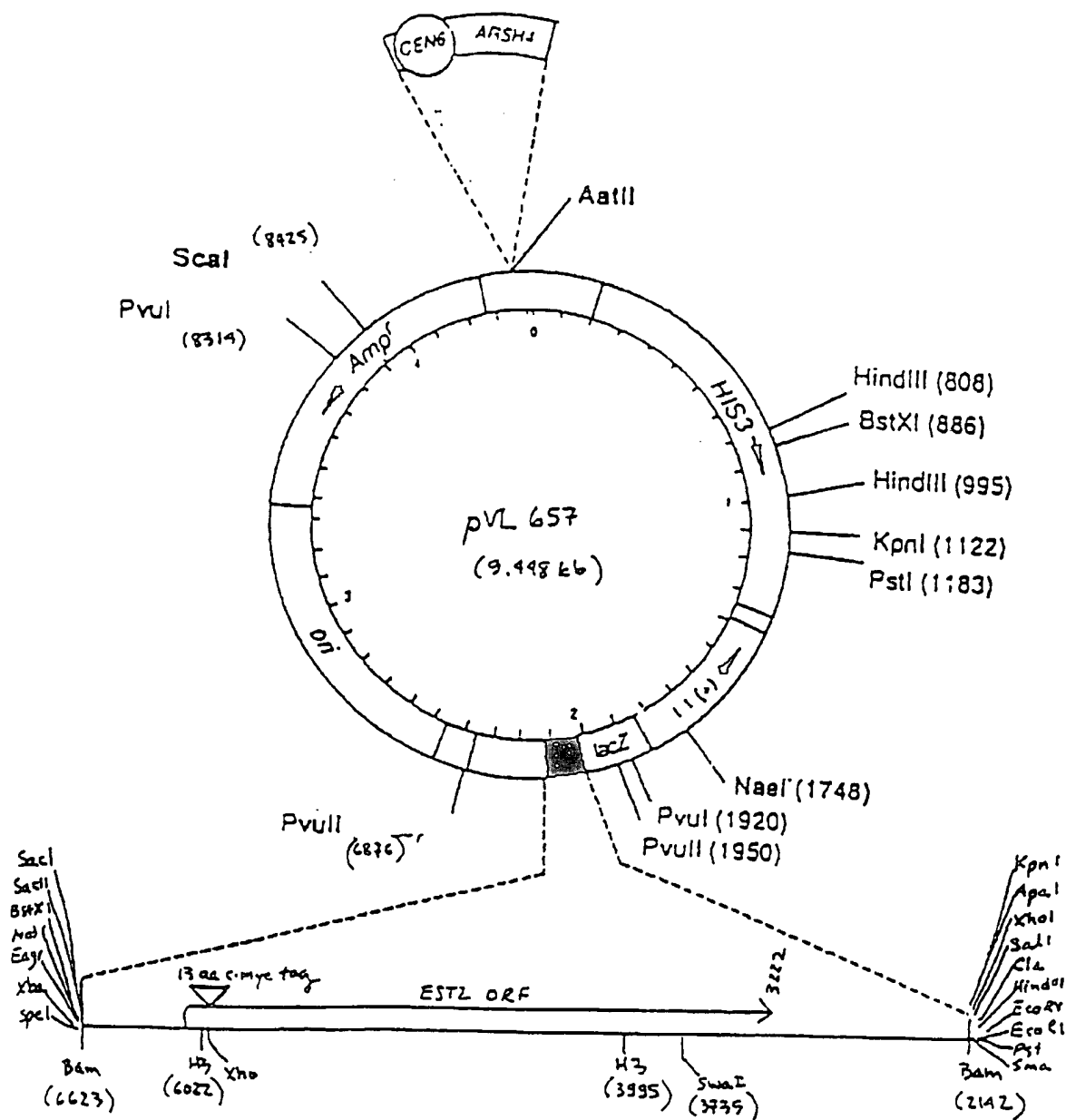
G. C-terminally (HA)<sub>3</sub> tagged EST3:

MPKVILESHSKPTDSVFLQPWIKALIEDNSEHDQYHPSGHVIPSLTKQDLALPHMSPTILT  
NPCHFAKITKFYNVCDYKVYASIRDSSHQILVEFSQECVSNFERTHNCRITSETTNCLMII  
GDADLVYVNSRAMSHFKICLSNISSKEIVPVLNVNQATIFDIDQVGSLSLTFPFVYKYL GSM  
YPYDVDPDYAYPYDVDPDYARSMYPYDVDPDYASLGGPRSKLKNM

H. baculovirus-expressed c-terminally (His)<sub>6</sub> tagged,  
frameshift corrected

MPKVILESHSKPTDSVFLQPWIKALIEDNSEHDQYHPSGHVIPSLTKQDLALPHMSPTILT  
NPCHFAKITKFYNVCDYKVYASIRDSSHQILVEFSQECVSNFERTHNCRITSETTNCLMII  
GDADLVYVNSRAMSHFKICLSNISSKEIVPVLNVNQATIFDIDQVGSLSLTFPFVYKYLHHH  
HHH





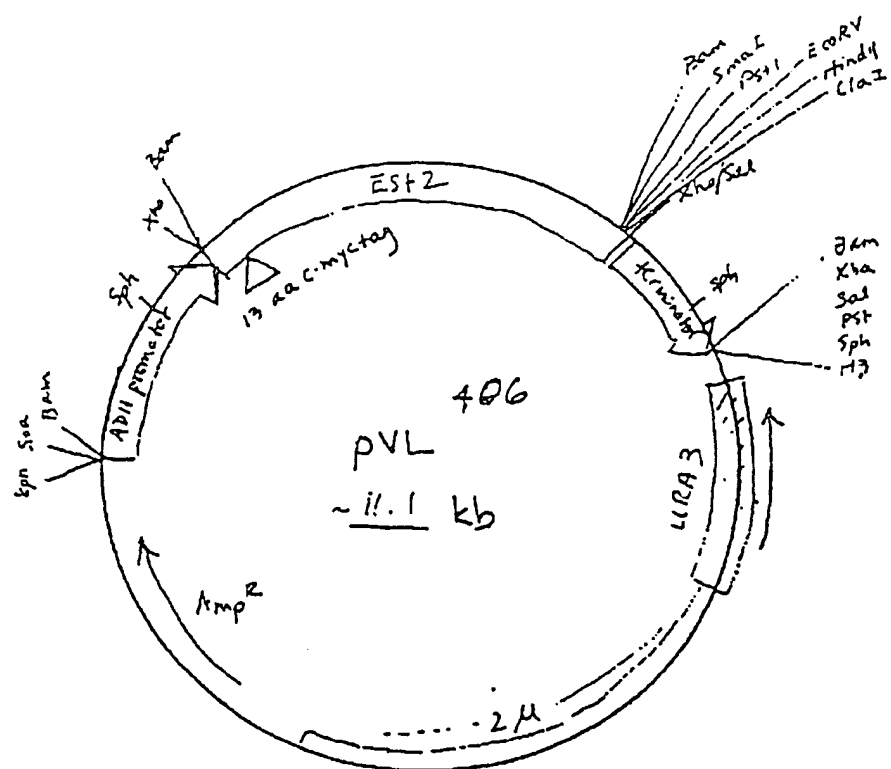


Fig. 14

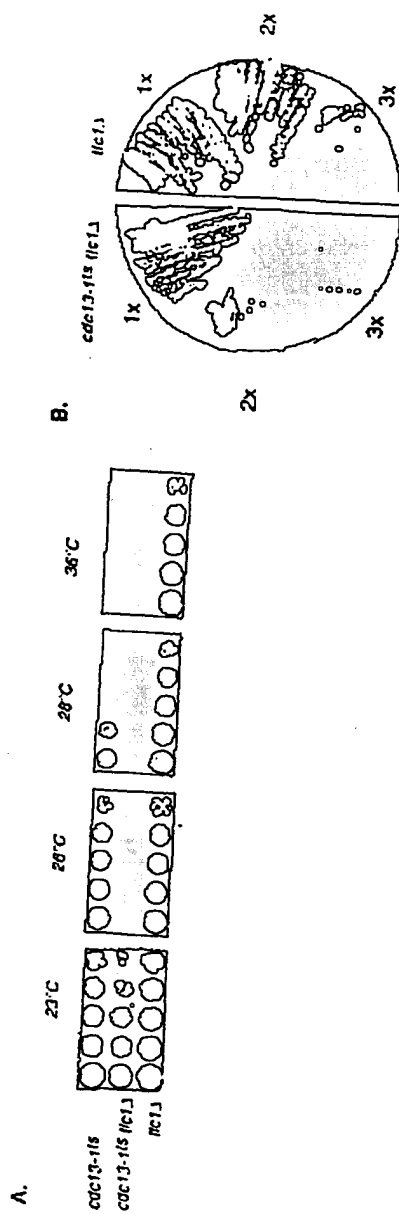
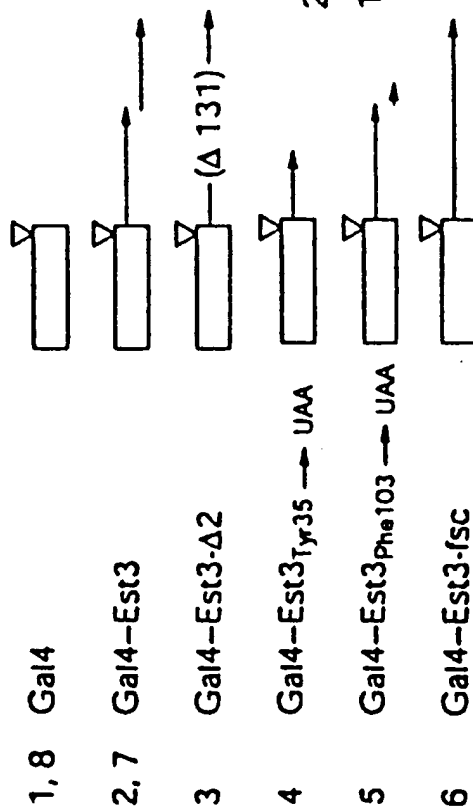


Figure 15

A



B

